VASCULAR FUNCTION, EXERCISE-INDUCED HYPEREMIA, AND ANTIOXIDANT SUPPLEMENTATION IN CHRONIC HEART FAILURE PATIENTS AND HEART TRANSPLANT RECIPIENTS

by

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STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The overall objective of this dissertation was to provide greater insight into changes in vascular function, blood flow regulation, and exercise-induced hyperemia associated with health, chronic heart failure (CHF), and heart transplantation (HTx). In the first study, we aimed to determine the central and peripheral contributions to movement-induced hyperemia in response to passive movement by comparing humans with a denervated heart (HTx) to intact controls. We observed a four-fold reduction in the transient increase in femoral blood volume entering the leg in response to passive limb movement in the HTx recipients compared to controls. These findings highlight the key role of the reflex increases in heart rate (HR) and the associated rise in cardiac output (CO) response as an important mechanism which contributes to movement-induced hyperemia in humans. The second study investigated the changes in vascular function and the role of oxidative stress from health to CHF, HTx, and beyond. Utilizing flow-mediated vasodilation (FMD) we documented reduced vasodilatory capacity in CHF patients, which was normalized in early HTx recipients, and then an eventual decline in vascular function in the HTx recipients that were the furthest time from transplantation (>14 yrs post-HTx). The acute ingestion of the antioxidant cocktail (AOC) was able to significantly increase FMD by 55% in these >14 yrs post-HTx recipients suggesting that free radicals, and the associated decrease in nitric oxide bioavailability, are largely

responsible for their endothelial dysfunction. The third study sought to better characterize the role of free radicals in regulating central and peripheral hemodynamics at rest and during exercise in patients with CHF using an oral AOC and dynamic handgrip exercise. The ingestion of the AOC had significant systemic hemodynamic effects which were only evident in the patients with CHF. Specifically, the AOC further reduced the patients already lower MAP (~5%), increased CO (~10%), and caused a fall in systemic vascular resistance (~12%). These data imply that systemic vascular resistance appears, at least in part, to be free radically-mediated. Collectively, this research has provided significant insight into the cardiovascular consequences of CHF and HTx in terms of oxidative stress, vascular function and hemodynamic regulation.

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CHAPTER 1

INTRODUCTION

Chronic heart failure (CHF) is a major cause of morbidity and mortality in the United States and according to the American Heart Association (AHA) is the leading cause of hospitalization among those individuals on Medicare (11). The AHA also estimates that more than 5 million people in the United States have CHF, with 550,000 new cases being diagnosed each year. CHF is a disease that has many different origins and is typically the most severe manifestation of almost every form of cardiac disease, including myocardial infarction, coronary atherosclerosis, hypertension, congenital heart disease, and cardiomyopathies.

The underlying physiology of CHF is the inability of the heart to fill or pump blood efficiently, compromising systemic perfusion, increasing sympathetic nerve activity, and limiting physical function. CHF most often effects older individuals that have one or more of the following risk factors: high blood pressure, coronary artery disease, myocardial infarction, irregular heartbeats, diabetes, congenital heart defects, sleep apnea, certain viruses, alcohol use, or some kidney conditions. There is no cure for CHF and in the most severe stage of this disease process; heart transplantation (HTx) is usually the best option.

In recent decades heart transplantation has advanced from an experimental procedure to an accepted life-extending therapy for patients with the most advanced stages of heart failure. The health and elasticity of major arteries is a risk factor for various types of cardiovascular disease (4) and has also been suggested to "naturally" deteriorate with age (12). Therefore, as quality of life and survival rates continue to climb for HTx patients, there is greater interest in understanding the long term effects of

HTx and the associated pharmacological therapy, particularly in the areas of vascular function and blood flow regulation.

HTx not only removes the failing organ but also results in denervation of the heart, which means there is no longer direct parasympathetic or sympathetic cardiac control. Additionally, many of these patients had a chronic debilitating cardiac illness preoperatively, resulting in many detrimental physiological changes. Vascular dysfunction and blood flow dysregulation have been documented in patients with CHF (9), resulting from the inability of vessels to respond to physiological stimuli such as shear stress (6) and augmented sympathetic nerve activity (5). Thus, the ability to respond and adapt to changing stimuli, such as exercise, may be very different in both CHF patients and HTx recipients compared to healthy individuals.

The understanding and monitoring of vascular function is important as it relates to the prevalence of cardiovascular disease (7) and is even more germane in patients with CHF and following HTx. Additionally, in light of the increasing interest in isolating the physiological and mechanical contributors to the blood flow response, especially during exercise (exercise hyperemia) (16), the abnormal hemodynamic responses common in CHF and HTx patients may help to further explain the mechanisms responsible for vessel vasodilation and exercise hyperemia in both health and disease.

Oxidative stress is caused by an imbalance between free radical production and antioxidant capacity. High levels of free radicals have been associated with many chronic diseases including hypertension, atherosclerosis, diabetes, and the normal aging process (17), but this is especially true for CHF (1, 8). In CHF patients, elevated free radicals have been linked to impaired endothelial-dependent vasodilation (13, 14) and exaggerated

sympathetic nerve activity particularly with exercise (10, 15). In HTx recipients, the development of high levels of oxidative stress has also been associated with endothelial dysfunction (2) and the development of cardiac transplant-associated arteriosclerosis (3). This, coupled with the highly invasive nature of heart transplantation and the effects of previous cardiac illness, lends itself to the idea that antioxidant therapy may decrease circulating levels of free radicals and may actually improve vascular function and blood flow regulation in HTx recipients.

Accordingly, vascular function, blood flow regulation, and exercise-induced hyperemia will be examined in healthy controls, CHF patients, and HTx recipients with varied time since surgery with and without oral antioxidant supplementation. Specifically, the first study will utilize HTx as a denervated model to better understand the central and peripheral contributions to exercise-induced hyperemia. The second study will take a more clinically relevant approach comparing vascular function, assessed by flow-mediated vasodilation (FMD) and reactive hyperemia (RH) with and without the supplementation of an antioxidant cocktail in healthy controls, CHF patients, and HTx recipients. The third study will determine the role of oxidative stress in the central and peripheral responses to handgrip exercise for healthy controls and patients with CHF. The overall objective of this dissertation will be to provide greater insight into changes in vascular function, blood flow regulation, and exercise-induced hyperemia associated with health, CHF, and HTx.

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CHAPTER 2

UNDERSTANDING EXERCISE-INDUCED HYPEREMIA: CENTRAL AND PERIPHERAL HEMODYNAMIC RESPONSES TO PASSIVE LIMB MOVEMENT IN HEART TRANSPLANT RECIPIENTS

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Understanding exercise-induced hyperemia: central and peripheral hemodynamic responses to passive limb movement in heart transplant recipients

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[1]*Geriatric Research Education and Clinical Center and* [2]*Division of Cardiology, Department of Internal Medicine, George E. Whalen Veterans Affairs Medical Center, Salt Lake City;* [3]*Division of Geriatrics, Department of Internal Medicine, and* [4]*Department of Exercise and Sport Science, University of Utah, Salt Lake City, Utah*

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Hayman MA, Nativi JN, Stehlik J, McDaniel J, Fjeldstad AS, Ives SJ, Wray DW, Bader F, Gilbert EM, Richardson RS. Understanding exercise-induced hyperemia: central and peripheral hemodynamic responses to passive limb movement in heart transplant recipients. *Am J Physiol Heart Circ Physiol* 299: H1653–H1659, 2010. First published September 10, 2010; doi:10.1152/ajpheart.00580.2010.—To better characterize the contribution of both central and peripheral mechanisms to passive limb movement-induced hyperemia, we studied nine recent (<2 yr) heart transplant (HTx) recipients (56 ± 4 yr) and nine healthy controls (58 ± 5 yr). Measurements of heart rate (HR), stroke volume (SV), cardiac output (CO), and femoral artery blood flow were recorded during passive knee extension. Peripheral vascular function was assessed using brachial artery flow-mediated dilation (FMD). During passive limb movement, the HTx recipients lacked an HR response (0 ± 0 beats/min, Δ0%) but displayed a significant increase in CO (0.4 ± 0.1 l/min, Δ5%) although attenuated compared with controls (1.0 ± 0.2 l/min, Δ18%). Therefore, the rise in CO in the HTx recipients was solely dependent on increased SV (5 ± 1 ml, Δ5%) in contrast with the controls who displayed significant increases in both HR (6 ± 2 beats/min, Δ11%) and SV (5 ± 2 ml, Δ7%). The transient increase in femoral blood volume entering the leg during the first 40 s of passive movement was attenuated in the HTx recipients (24 ± 8 ml) compared with controls (93 ± 7 ml), whereas peripheral vascular function (FMD) appeared similar between HTx recipients (8 ± 2%) and controls (6 ± 1%). These data reveal that the absence of an HR increase in HTx recipients significantly impacts the peripheral vascular response to passive movement in this population and supports the concept that an increase in CO is a major contributor to exercise-induced hyperemia.

blood flow; skeletal muscle

THE TRANSITION FROM REST to exercise evokes an increase in skeletal muscle blood flow that matches oxygen supply to the increased metabolic demand. There are various central and peripheral mechanisms that are likely responsible for this exercise-induced hyperemia. These include the skeletal-muscle pump (29, 42), cardioacceleration induced by muscle mechanoreceptor and metaboreceptor feedback (1, 2), as well as mechanical (8, 24, 45) and flow-mediated vasodilation (26, 34). However, there is currently little accord as to the contribution, timing, or magnitude of the central versus the peripheral hemodynamic responses to exercise-induced hyperemia in humans.

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Using second-by-second temporal analysis of femoral blood flow, cardiac output (CO), heart rate (HR), and stroke volume (SV), our group (30) recently reported that passive leg extension resulted in an HR-driven increase in CO. This contrasts with previous research (14, 51) that either reported or assumed no change in CO with this passive exercise model. Indeed, we suggested that the increase in HR and the resulting rise in CO are a primary initiator of movement-induced hyperemia (30). Additionally, we observed that a significant increase in CO occurred at the onset of passive exercise even when blood flow to the exercising leg was prevented via cuff occlusion, revealing that an increase in leg blood flow is not obligatory for an increase in CO. Although these data further support a distinct contribution of both peripheral and central hemodynamic factors to exercise-induced hyperemia, the failure to manipulate the CO response still leaves one unable to clearly separate the two.

Heart transplant (HTx) results in heart denervation, a unique circumstance where both afferent and efferent cardiac control mechanisms are interrupted. It is well documented that heart transplantation limits the central hemodynamic response to exercise (19, 22, 38), providing a model through which it is possible to separate the contributions of central and peripheral mechanisms to passive movement-induced blood flow. Additionally, exercise tolerance of HTx recipients is reduced compared with healthy individuals (19, 35), which is particularly true in the first years following transplantation (17, 41). Therefore, a better understanding of the challenges to be overcome as the recipients transition from rest to exercise could in turn lead to an identification of rehabilitative approaches that might result in a faster recovery of exercise tolerance after HTx and an improved quality of life.

Consequently, we sought to use HTx recipients as a model devoid of cardiac innervation to further delineate central and peripheral contributions to exercise-induced hyperemia. Using passive leg movement in both healthy and HTx recipients, we hypothesized that *1*) in HTx recipients, passive limb movement will not increase HR and CO as it does in healthy, age-matched controls, and *2*) as a CO increase is an important contributor to exercise-induced hyperemia, movement-induced increases in leg blood flow will be significantly attenuated in the HTx recipients.

METHODS

Subjects

Nine HTx recipients (<2 yr post-HTx) and nine healthy controls were recruited in the HTx clinic at the University of Utah and the Salt

Table 1. *Subject characteristics*

	Control	HTx
Male/Female	8/1	8/1
Age, yr	58 ± 5	56 ± 4
Weight, kg	85 ± 4	85 ± 10
Height, cm	178 ± 2	177 ± 7
Body mass index, kg/m^2	27 ± 1	24 ± 3
Systolic blood pressure, mmHg	129 ± 5	127 ± 8
Diastolic blood pressure, mmHg	75 ± 3	78 ± 7
Glucose, mg/dl	93 ± 2	107 ± 12
Cholesterol, mg/dl	187 ± 13	145 ± 15
HDL, mg/dl	50 ± 5	39 ± 3
LDL, mg/dl	122 ± 12	86 ± 12
Triglycerides, mg/dl	97 ± 17	132 ± 18
Hemoglobin, g/dl	15 ± 0.3	12 ± 0.5*
WBC, K/μl	5.5 ± 0.5	5.5 ± 0.7
Neutrophil, K/μl	3.3 ± 0.5	4.5 ± 0.7
Lympocyte, K/μl	1.6 ± 0.1	0.6 ± 0.1
Monocyte, K/μl	0.4 ± 0.0	0.4 ± 0.1

Values are means ± SE. HTx, heart transplant; HDL, high-density lipoprotein; LDL, low-density lipoprotein; WBC, white blood cells. *Significantly different from control.

Lake City Veterans Affairs Medical Center. Additional subject characteristics and the therapy of the HTx recipients at the time of the study are reported in Tables 1 and 2. The protocol was approved by and written informed consent was obtained according to the Institutional Review Board of the University of Utah and the Salt Lake City Veterans Affairs Medical Center. All studies were performed on the same day in a thermoneutral environment. Subjects reported to the laboratory in the fasted state and had not performed any exercise within the past 24 h.

Brachial Artery Flow-Mediated Dilation and Reactive Hyperemia Protocol

Subjects rested supine for ~20 min, and a blood pressure cuff was placed on the upper right arm proximal to the elbow but distal to the placement of the ultrasound Doppler probe on the brachial artery. Baseline measurements were obtained, and the arm cuff was inflated to a suprasystolic pressure (>250 mmHg) for 5 min. Measurements of brachial artery diameter and blood velocity were collected continuously for 2 min following cuff deflation.

Passive Exercise Protocol

Subjects again rested supine for ~20 min before the start of the data collection and remained in this position throughout the entire protocol. The protocol consisted of a 60-s resting baseline data acquisition followed by a 3-min bout of passive leg extension. Before the start of baseline and passive movement, a cuff was placed distal to the knee on the passive leg and inflated to 250 mmHg, eliminating blood flow to the lower leg for the entire 3 min. This was done to eliminate fluctuations in blood flow to the lower leg as a consequence of changing gravitational and centrifugal forces on the lower leg throughout the movement. Initial pilot work revealed a minimal effect of either cuffing or not cuffing the control leg in the same manner. Therefore, a lower leg cuff on the control leg was not applied in this study. Passive exercise was achieved by a member of the research team moving the subject's lower leg through a range of motion, defined by 90° and 180° knee joint angles, at a rate of 1 Hz (throughout the protocol, the control leg remained fully extended and supported). Real-time feedback to the investigator was provided by a position sensor to ensure a consistent range of motion, and a metronome was used to maintain the cadence. Before the start and throughout the protocol, the subjects were encouraged to remain passive and resist any urge to assist with leg movement. To avoid a startle reflex and active resistance to the passive movement, the subjects were made aware that passive movement would take place, but, to minimize the chance of an anticipatory response, they were not informed of exactly when this movement would initiate.

Measurements

Arterial blood flow and blood velocity measurements and analyses. Measurements of arterial blood velocity and vessel diameter were performed in both the brachial artery flow-mediated dilation (FMD) and passive exercise protocols with Logic 7 and Logic e ultrasound systems (General Electric Medical Systems, Milwaukee, WI). The Logic 7 and Logic e were equipped with linear array transducers operating at an imaging frequency of 14 and 12 MHz, respectively. Vessel diameter was determined at a perpendicular angle along the central axis of the scanned area. Blood velocity was obtained using the same transducers with a Doppler frequency of 5 MHz. All blood velocity measurements were obtained with the probe appropriately positioned to maintain an insonation angle of 60° or less. The sample volume was maximized according to vessel size and was centered within the vessel based on real-time ultrasound visualization. Arterial diameter was measured, and mean velocity (V_{mean}) values [angle-corrected and intensity-weighted area under the curve (AUC)] were then automatically calculated using commercially available software (Logic 7 and Logic e). Using arterial diameter and V_{mean}, the blood flow in the brachial and femoral arteries was calculated as follows: Blood flow = $V_{mean}\pi$(vessel diameter/2)$^2 \times 60$, where blood flow is in milliliters per minute.

Flow-mediated dilation. Relative and absolute FMD were calculated as the percent change and the absolute Δ, respectively, from resting artery diameter to the largest diameter achieved during the 120 s of postinflation imaging. All ultrasound vessel lumen diameter measurements were evaluated during end diastole (corresponding to an R wave documented by the simultaneous ECG signal) (Logic 7). An analysis of the diameters was performed using off-line automatic edge-detection Brachial Analyzer software (Medical Imaging Applications, Coralville, IA), which is described in detail elsewhere (33a).

Shear rate and reactive hyperemia calculation. Shear stress is believed to be the mechanism that stimulates the vascular endothelium and results in subsequent vasodilation (7). Since blood viscosity was not measured, shear rate, an adequate surrogate measure (4, 36), was calculated using the following equation: Shear rate (in s^{-1}) = 8·V_{mean} (in cm/s)/vessel diameter (in cm). Cumulative reactive hyperemia (RH) (AUC) post-cuff release (total blood flow over 2 min) was integrated using the trapezoidal rule and calculated as follows: $\sum[y_i(x_{i+1} - x_i) + (1/2)(y_{i+1} - y_i)(x_{i+1} - x_i)]$. To normalize the vasodilation for shear rate, FMD was divided by the cumulative shear rate (%Δdiameter/s^{-1}·s) (37).

Table 2. *Characteristics pertinent to the HTx recipient group*

	HTx
n	9
Diagnosis (nonischemic cardiomyopathy)	4/9
Diagnosis (ischemic cardiomyopathy)	5/9
Time post-HTx, months ± SE	9 ± 2
Rejection episodes	1/9
Left ventricular ejection fraction, %	65.3 ± 1.8
Medications, number of all cases	
Cyclosporine	1/9
Tacrolimus	7/9
Azathioprine	3/9
Mycophenolic acid	5/9
Prednisone	4/9
β-Blockers	1/9

Central variables. HR, SV, CO, and mean arterial pressure (MAP) were determined with a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands). SV was calculated using the Modelflow method that includes age, sex, height, and weight in its algorithm (Beatscope version 1.1; Finapres Medical Systems) (5) and has been shown to accurately track CO during a variety of experimental protocols including exercise (9, 10, 44, 44a, 46). CO was then calculated as the product of HR and SV. Vascular conductance within each leg was calculated as leg blood flow/MAP.

Data acquisition. Throughout the entire protocol, HR, SV, CO, MAP, ECG, and knee joint angle signals underwent analog-to-digital conversion and were simultaneously acquired (200 Hz) using commercially available data acquisition software (AcqKnowledge, Biopac Systems). In addition, this data acquisition software also acquired (10,000 Hz) the audio anterograde and retrograde signals from both Doppler ultrasound systems to serve as a qualitative indicator of blood velocity changes and to ensure an accurate temporal alignment of blood velocity measurements obtained from these systems and the other signals collected (i.e., finometer and goniometer) (30).

Statistical Analyses

Statistics were performed using commercially available software (SPSS, v. 17.0, Chicago, IL). An independent *t*-test ($\alpha < 0.05$) was used to compare FMD and RH in HTx recipients and controls. As both central and peripheral responses were transient, only data from the first 40 s of passive movement were compared, and before analysis all data were smoothed using a rolling 3-s average. A paired *t*-test ($\alpha <$

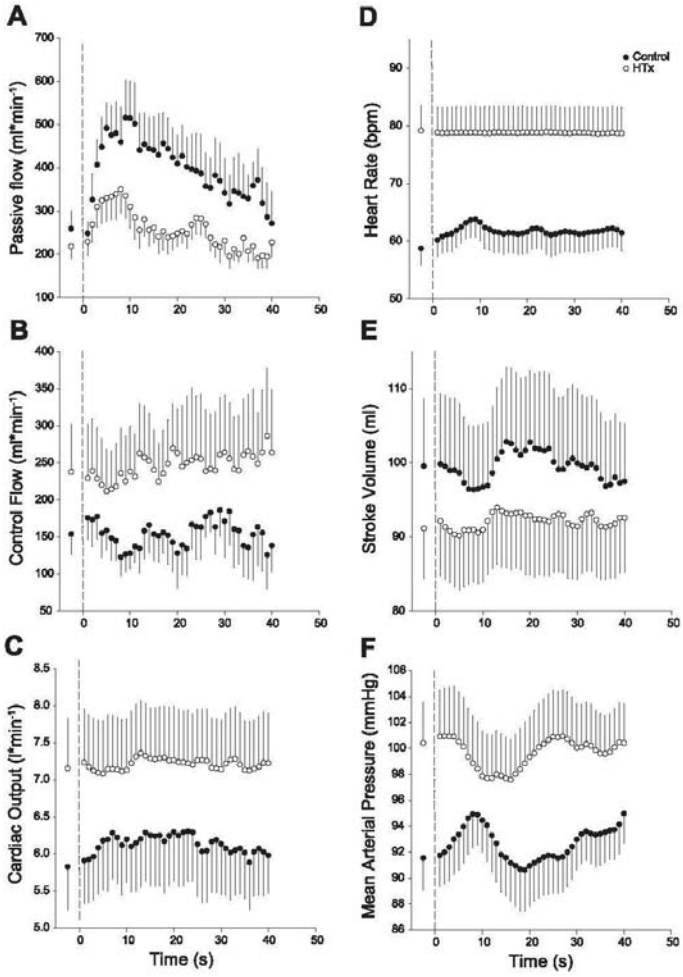

Fig. 1. Central and peripheral hemodynamic responses to passive exercise in heart transplant (HTx) recipients and controls. Values are means ± SE of passive leg blood flow (*A*), control leg blood flow (*B*), cardiac output (CO; *C*), heart rate (HR; *D*), stroke volume (SV; *E*), and mean arterial pressure (MAP; *F*) for the first 40 s of the exercise protocol. bpm, Beats/min. The transition from rest to movement occurs at 0 on the axis. Note: these figures are presented to illustrate the general trends. As the analyses were performed on data from individuals who exhibited varying response kinetics, averaging removes some of the information in the original individual recordings. Therefore, maximum change tends to be underestimated here but is represented in Fig. 2.

0.05) was used to compare whether individual maximal relative changes in CO, HR, SV, and blood flow differed from baseline within each group, and an independent *t*-test ($\alpha < 0.05$) was used to identify differences between healthy controls and HTx recipients. A paired *t*-test ($\alpha < 0.05$) was also used to compare control leg blood flow responses from the passively moved leg within each group. All group data are expressed as means ± SE.

RESULTS

Peripheral Responses to Passive Exercise

The peripheral blood flow responses to passive movement over time are illustrated in Fig. 1, *A* and *B*, and all maximum changes from baseline are represented in Fig. 2. Baseline blood flows were not different between HTx recipients (218 ± 29 ml/min; 237 ± 65 ml/min) and controls (259 ± 41 ml/min; 154 ± 29 ml/min) in the passive and control leg, respectively. Following the onset of passive movement, the maximum blood flow achieved in the passively moved leg increased significantly above baseline in both HTx recipients (397 ± 53 ml/min, Δ85%) and controls (623 ± 78 ml/min, Δ156%) although attenuated in the HTx recipients (Fig. 2). In the HTx recipients, the percent change from baseline (Fig. 2) in the passively moved leg was not significantly different from the control leg, unlike the control group where there was a significant difference between legs. The transient increase in femoral blood flow, as assessed by the AUC during the first 40 s of passive limb movement, resulted in a smaller blood volume entering the leg in the HTx recipients (24 ± 8 ml) compared with the controls (93 ± 25 ml).

Central Responses to Passive Exercise

The central responses to passive movement over time are illustrated in Fig. 1, *C–F*. Baseline values for CO and SV were not significantly different between the HTx recipients and controls; however, there were baseline differences in HR ($P = 0.001$) and MAP ($P = 0.05$). All maximum changes from baseline are represented in Fig. 2. The HTx recipients displayed a significant increase in CO (0.4 ± 0.1 l/min, Δ5%) although greatly attenuated, compared with the controls (1.0 ± 0.2 l/min, Δ18%). In the HTx recipients, there was an absence of a significant increase in HR (0 ± 0 beats/min, Δ0%). Therefore, the CO response was solely dependent on increased SV (5 ± 1 ml, Δ5%). These central responses in the HTx recipients were accompanied by a significant decrease in MAP (−5 ± 1 mmHg, Δ−5%). In contrast, the controls who significantly increased HR (6 ± 2 beats/min, Δ11%) as well as SV (5 ± 2 ml, Δ7%) exhibited an increase in MAP (7 ± 1 mmHg, Δ7%) (Fig. 2). The maximal changes in vascular conductance in the passive leg were greater in the controls (3 ± 1 ml·min^{-1}·mmHg^{-1}, Δ156%) compared with the HTx recipients (2 ± 0.3 ml·min^{-1}·mmHg^{-1}, Δ87%).

Vascular Function: FMD and RH

There was no significant difference in peripheral vascular function between the HTx recipients and controls as assessed by FMD (Fig. 3). This was the case whether FMD was expressed in traditional terms (%diameter change) (controls, 6 ± 1%; and HTx recipients, 8 ± 2%) or normalized for shear rate (FMD/shear rate) (controls, 0.1 ± .02; and HTx recipients, 0.2 ± 0.06). Resting brachial artery blood flows did not differ between controls and HTx recipients (110 ± 15 and 90 ± 11 ml/min, respectively) (Fig. 4). Similarly, RH, both in terms of peak (controls, 805 ± 64 ml/min; and HTx recipients, 680 ± 64 ml/min) and AUC (controls, 627 ± 69 ml; and HTx recipients, 564 ± 58 ml), was not different between groups (Fig. 4).

DISCUSSION

In this study, we sought to determine the central and peripheral contributions to movement-induced hyperemia in response to passive movement by comparing humans with a denervated heart (HTx) to intact controls. With this approach, we observed a fourfold reduction in the transient increase in femoral blood volume entering the leg in response to passive limb movement in the HTx recipients compared with controls. This attenuated hyperemic response to movement in the HTx recipients was not likely due to diminished peripheral vascular function, as measured by brachial artery FMD and RH, implicating a differing central hemodynamic response as the source of disparity in leg blood flow. These findings highlight the key role of the reflex increases in HR and the associated rise in CO response as an important mechanism that contributes to movement-induced hyperemia in humans.

Central and Peripheral Responses to Passive Limb Movement

Almost instantaneously upon the initiation of leg movement in the control subjects, there was a significant increase in HR (Fig. 1), which was likely due to the stimulation of peripheral muscle and joint afferent reflexes (1, 2, 32) and subsequent vagal inhibition (33). This HR increase contributed to the

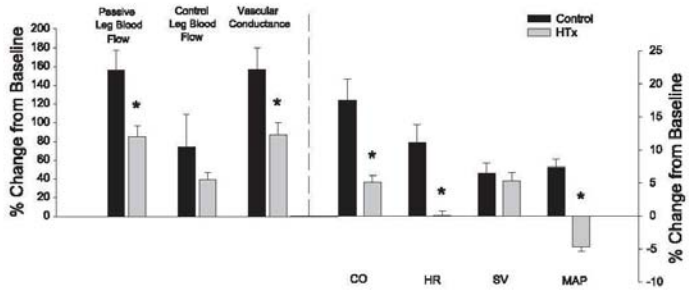

Fig. 2. Maximum relative changes in passive leg blood flow, control leg blood flow, vascular conductance, CO, HR, SV, and MAP in HTx recipients and controls. Values are means ± SE. *Significantly different from control. Note: *y*-axis scale for the blood flow data is different from *y*-axis scale for the central variables (dashed vertical line separates the variables associated with the *y*-axis scales on *left* and *right*).

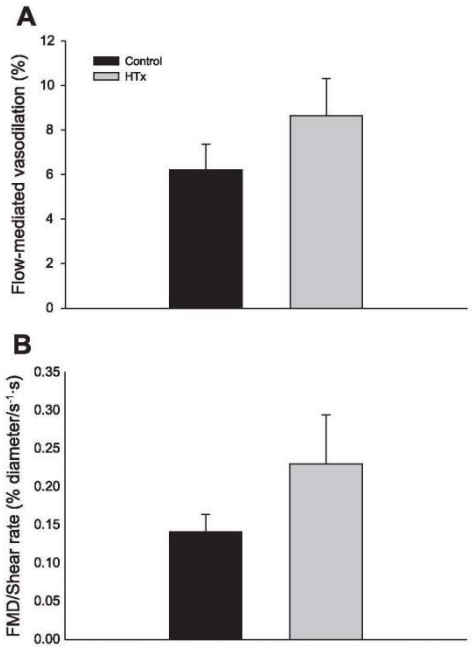

Fig. 3. Brachial artery flow-mediated dilation (FMD) following 5-min cuff occlusion expressed as a percent change from precuff baseline (*A*) and after normalizing FMD for shear rate (*B*) in HTx recipients and controls. Values are means ± SE. There were no significant differences.

increase in CO (≈18%) in the control subjects. The timing and magnitude of both these responses was similar to recent results published by our group (30), which suggested that an HR-driven increase in CO is a primary initiator of movement-induced hyperemia. Despite baseline values that were not statistically different, there was no HR response to passive limb movement in the HTx group, which confirmed the appropriateness of using HTx recipients as a model devoid of a chronotropic response (Fig. 1). Interestingly, HTx recipients also exhibited a significant increase in CO (≈ 5%) with passive limb movement. When compared with that of the normal controls, this CO response was reduced in magnitude and the timing of the CO increase was delayed by 11 to 12 s. This delay related to the fact that the CO increase in HTx patients was solely dependent on an increase in SV rather than HR.

Previous studies have suggested that an increase in limb blood flow and the resultant rise in venous return could be the predominant mechanism responsible for an increase in CO with passive limb movement (14). In the present study, there was no difference in the magnitude of SV increases between the controls and the HTx recipients and, as already noted, the SV response was similarly delayed in both groups, perhaps indicative of the transit time for the increased blood volume to travel through the passively moved leg (3) and then return back to the heart. Although long-established hemodynamic principles demand that an increase in venous return is required to sustain an increase in CO, the temporal relationships between SV, HR, and CO documented in this study suggest that in the first few seconds of limb movement, there may be a mismatch between CO and venous return, such that the initial increase in CO can be attributed to the almost instantaneous increase in HR. This observation is emphasized here by the lack of an HR response and a subsequently delayed and attenuated CO increase in the HTx recipients (Fig. 1).

Upon initiation, passive leg movement consistently results in an increase in femoral blood flow (14, 30, 51). As hypothesized, hyperemia in the passively moved leg was significantly attenuated by approximately fourfold in the HTx recipients (Figs. 1 and 2), and much of the observed difference in maximum blood flow between the controls (≈ 156%) and the HTx recipients (≈ 85%) could be attributed to the lack of an HR increase and the concomitantly diminished CO response. The differing hyperemic response between these groups further supports the previous conclusions from our group (30) that an HR-driven increase in CO is an essential component of the hyperemic response at the onset of leg movement and that limb vasodilation and the subsequent venous return are not solely responsible for the increased CO in the first few seconds following limb movement.

MAP and Vascular Conductance

During passive leg movement, there was an increase in MAP (≈7 mmHg) in the control subjects, an expected effect of an increase in CO and the concomitant change in vascular conductance. However, this intuitive finding is in contrast with our previous study (30), which reported a small decrease in MAP (≈−3 mmHg) upon initiation of passive leg movement. This discrepancy may be a result of age-related differences in vascular function and/or structure (6, 28, 31, 48) and baroreceptor reflexes that previously minimized this potential for a rise in blood pressure with an increase in CO as the present control group was older (≈58 yr) compared with the group in our previous study (≈33 yr). Thus perhaps baroreflex control of HR in response to limb movement plays a more important role in young rather than older subjects. In contrast, MAP in the HTx recipients exhibited a comparable magnitude of

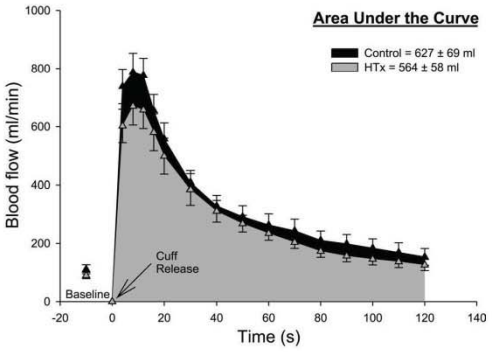

Fig. 4. Resting brachial artery blood flow and reactive hyperemia following cuff occlusion in healthy controls and HTx recipients.

change to the current age-matched controls but in the opposite direction (≈−5 mmHg). This was most likely a consequence of a drop in peripheral resistance by mechanically induced vessel dilation (8, 24) and FMD (25, 26, 34), allowing blood flow to increase to the passively moved limb but without the same magnitude of CO increase documented in the controls to either raise or successfully maintain MAP. Despite the contrasting and significant changes in MAP, vascular conductance was qualitatively very similar to blood flow with the maximal change being far greater in the controls compared with the HTx recipients (Fig. 2). Thus the difference in hyperemia observed between the two groups could not be explained solely by changes in MAP.

Peripheral Vascular Function

Differences in the hyperemic response to leg movement between the HTx recipients and controls could conceivably be attributed to differences in peripheral vascular function as a result of the prior disease state, independent of central cardiac differences. Peripheral vascular dysfunction is known to occur in chronic heart failure (11, 12, 20, 21). However, somewhat surprisingly, there is evidence that endothelial function improves within the first 12 mo following transplantation (27, 39). In agreement with this concept the peripheral vascular function of the current HTx recipients, as assessed by brachial artery FMD and RH, was not attenuated (Figs. 3 and 4). This further supports that the limited femoral blood flow response in this group was a result of the lack of an HR response and not peripheral vascular dysfunction. Although FMD and RH are just two of the possible measures of vascular function, these observations of vascular normalcy also support the use of the HTx patient model to elucidate the importance of HR and CO on movement-induced hyperemia.

Clinical Perspective

Following HTx, recipients exhibit substantial exercise intolerance (19, 35), particularly in the initial years following the procedure (17, 41). Many studies have demonstrated an association between exercise intolerance and chronotropic incompetence (15, 19, 38, 40). The HTx recipients in this study also demonstrated a lack of an HR response and an attenuated hyperemia at the initiation of passive limb movement. Although still controversial, there is accumulating evidence suggesting some sympathetic reinnervation occurs in HTx recipients based on a more normal chronotropic exercise response (15, 40), evidence of coronary norepinephrine spillover (43, 47), and HR variability (23). The passive limb movement approach used in this study helps to illustrate that other mechanisms besides HR (i.e., SV) increase CO in this population. Additionally, such a paradigm may provide clinicians and therapists with another tool with which to examine the impact and role of cardiac reinnervation without the added complexity of the changing metabolites associated with exercise.

Experimental Considerations

Although many subject characteristics and vascular function of the HTx recipients were not different from the controls, it must still be acknowledged that, in addition to the main reason for studying this population (a lack of cardiac innervation), the HTx recipients all previously had heart failure. Thus the recognized skeletal muscle dysfunction associated with this pathology may have influenced the results of this study; however, this issue was likely minimized by the fact that this study employed passive exercise which does not require a significant skeletal muscle metabolic response. An additional limitation of this study is the extrapolation of peripheral vascular function from the upper to the lower limbs, where the response to passive movement was actually assessed.

Summary

With the use of the denervated HTx patient model, these data provide evidence that an elevation in HR and the subsequent CO increase are an important contributor to the hyperemic response following the onset of passive limb movement. The attenuated blood flow response in the HTx recipients cannot easily be explained by differences in peripheral vascular function, as FMD and RH were not attenuated in the HTx recipients. These findings highlight the key role of the reflex increases in HR and the associated rise in CO response as an important mechanism for movement-induced hyperemia in humans.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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CHAPTER 3

EVIDENCE OF ALTERED VASCULAR FUNCTION AND THE ROLE OF OXIDATIVE STRESS IN HEART FAILURE, HEART TRANSPLANT, AND BEYOND

Abstract

Using flow-mediated vasodilation (FMD), reactive hyperemia (RH), and an acute oral antioxidant cocktail (AOC (Vitamin C, E + α lipoic acid)), this study aimed to provide greater insight into altered vascular function and the role of oxidative stress with the development of chronic heart failure (CHF) and over time following heart transplant (HTx) surgery. Twelve healthy controls (58 ± 4 yrs), 14 CHF patients (62 ± 2 yrs), 12 recent (< 3 yrs post) HTx recipients (58 ± 3 yrs), 12 (5-10 yrs post) HTx recipients (59 ± 4 yrs), and 11 (> 14 yrs post) HTx recipients (67 ± 2 yrs), ingested either placebo (PL) or AOC prior to FMD and RH testing. Vascular function, as measured by FMD, was not different between the controls (6.8 ± 1.9 %), recent < 3 yrs post-HTx group (8.1 ± 1.2%), and the 5-10 yrs post-HTx group (5.5 ± 1.0%). However, PL FMD was lower in the CHF patients (4.5 ± 0.7%) and in the > 14 yrs post-HTx group (2.9 ± 0.8%). The AOC increased plasma ascorbate levels in all groups, but only increased FMD in the controls (PL 6.8 ± 1.9%; AOC 9.2 ± 1.0%) and > 14 yrs post-HTx recipients (PL 2.9 ± 0.8%; AOC 4.5 ± 1.3%), and RH had no effect in all groups. Thus, vascular function is blunted in CHF patients, normalizes soon after HTx surgery, but progressively declines to a level similar to CHF patients with free radicals implicated in this progression.

Introduction

Vascular endothelial dysfunction is a systemic pathology that impairs the health and vasomotor control of both conduit and resistance vessels. Impaired endothelium-dependent vasodilation has been associated with various cardiovascular diseases including hypertension (36), coronary artery disease (33), and CHF (10, 23), but more

importantly may also precede the development of these conditions (5, 6). Therefore, in these patient populations, as well as healthy individuals, endothelial dysfunction may be of prognostic value and consequently noninvasive tests such as flow-mediated dilation (FMD) (5, 6) and the measurement of reactive hyperemia (RH) (20, 32) have become common research tools for the assessment of vascular function. However, the real impact of disease progression and surgical intervention across the continuum from health, CHF, HTx, and beyond has not been well characterized.

CHF patients have chronically elevated peripheral vasoconstriction, the result of elevated sympathetic nervous system activity (17), the renin-angiotensin system (13), and a concomitant dysfunction of the peripheral vasculature (23). The latter appears to be the consequence of an attenuated L-arginine-nitric oxide pathway (24) and has been, at least partially, attributed to the increased destruction of nitric oxide (NO) by free radicals (1). Elevated levels of free radicals, particularly superoxide (O_2^-), contribute to decreased NO bioavailability (49) and the corresponding attenuation in endothelial function (8). Antioxidant supplementation, presumably by improving NO bioavailability, has previously restored endothelial function in healthy aged individuals (8, 12) as well as CHF patients (18). Interestingly, in CHF patients that have undergone a HTx, there is some evidence that endothelial function normalizes relatively soon following transplantation (26, 41), although this is still somewhat controversial (37, 43) and the role of free radicals in this apparent restoration of function soon after HTx and then beyond is unclear.

Accordingly, using FMD and RH to assess vascular function and an acute oral AOC to examine the role of free radicals, this study sought to provide greater insight into

changes in vascular function with the development of CHF, HTx, and beyond. We hypothesized that 1) when compared to controls, vascular function as assessed by FMD and RH, will be reduced in CHF patients, improved immediately following HTx, and progressively decline thereafter and 2) by attenuating the levels of free radicals, the ingestion of the AOC will improve vascular function in all groups with more pronounced effects in the CHF patients and the HTx recipients with a longer time post-surgery.

Methods

Subjects

A total of 61 subjects (12 healthy controls, 14 NYHA Class II and III CHF patients, and 35 HTx recipients (< 3 yrs post-HTx, 5-10 yrs post-HTx, and > 14 yrs post-HTx)) were recruited in the CHF and HTx clinics at the University of Utah and the Salt Lake City VA Medical Center. The protocol was approved by these institutions and written informed consent was obtained. Characteristics and the pharmacological therapy of the CHF and HTx recipients at the time of the study are reported in Tables 3 and 4. All studies were performed in a thermoneutral environment at least 3 days apart to allow for washout of the oral antioxidants. Subjects reported to the laboratory in the fasted state, and without caffeine or alcohol use for 12 and 24 hrs, respectively. They had not performed any exercise within the past 24 hrs and if antioxidants and/or a multivitamin were part of a subject's daily routine they were asked to refrain from such use for at least three days prior to testing days.

Brachial Artery Flow-mediated Dilation (FMD) and Reactive Hyperemia (RH)

Subjects rested supine for approximately 20 min, and a blood pressure cuff was placed on the upper right arm proximal to the elbow, but distal to the placement of the ultrasound Doppler probe on the brachial artery. Baseline measurements of brachial artery diameter and blood velocity were performed, and the arm cuff was inflated to a suprasystolic pressure (>250 mmHg) for 5 min. Brachial artery diameter and blood velocity were then assessed continuously for 2 min following cuff deflation.

Antioxidant Supplementation

Subjects received either the AOC or placebo in a balanced, single blind design for the subject's two visits. The supplements were administered 90 and 60 min prior to the FMD protocol. A split dosing was used to improve absorbance and distribution of the antioxidants. The first antioxidant dose included Vitamin E (200 IU), Vitamin C (500 mg), Alpha-lipoic Acid (300 mg), and the subsequent dose included Vitamin E (400 IU), Vitamin C (500 mg), and Alpha-lipoic Acid (300 mg). The placebo microcrystalline cellulose capsules, that were of similar taste, color and appearance, were also consumed in two equivalently timed doses. The efficacy of this AOC to reduce plasma free radical concentration has previously been established using ex-vivo spin trapping and electron paramagnetic resonance (EPR) spectroscopy (52).

Measurements

Ultrasound Doppler assessments

Brachial artery diameter and blood velocities were measured with Ultrasound Doppler using a General Electric (GE) Logiq 7 ultrasound system (GE Medical Systems, Milwaukee, WI, USA). This system was equipped with a linear array transducer operating at an imaging frequency of 12-14 MHz, with frequency selected to optimize image quality according to vessel depth (16). Blood velocity was obtained with the same transducer with a Doppler frequency of 5 MHz. An insonation angle of 60º or less was utilized and the sample volume was centered within the vessel and maximized according to vessel size. The same experienced sonographer performed all measurements.

Brachial artery diameter, blood velocity, and blood flow analyses

Analysis of arterial diameters were performed off-line using the automatic edge-detection Brachial Analyzer software (Medical Imaging Applications, LLC, Coralville, IA). Angle-corrected and intensity-weighted mean velocities (V_{mean}) were determined using commercially available software (Logic 7). Using V_{mean} and arterial diameter, brachial artery blood flow was calculated as: Blood flow = $V_{mean}\pi$ (arterial diameter/2)^2 x 60, where blood flow is measured in milliliters per min.

Shear rate and reactive hyperemia calculation

During an FMD test, laminar shear stress is believed to be the mechanism that stimulates the vascular endothelium and results in subsequent vasodilation of the brachial artery (53). However, as blood viscosity was not measured, shear rate, which can be an

adequate surrogate measure (39), was calculated using the following equation: Shear rate (s^{-1}) = $8V_{mean}$ /arterial diameter. Cumulative shear rate ($s^{-1} \cdot s$) (area under the curve, AUC) and the reactive hyperemia post cuff release (total blood flow over 2 min) were integrated using the trapezoidal rule and calculated as: $\sum(y_i(x_{(i+1)} - x_i) + (1/2)(y_{(i+1)} - y_i)(x_{(i+1)} - x_i))$.

Oxidative stress, and antioxidant assays

In both the placebo and antioxidant trials blood samples were obtained from the antecubital vein prior to FMD testing (1 hr following ingestion of the second dose of either AOC or placebo). Serum and plasma samples were stored at -80°C until analysis. Quantitative determination of thiobarbituric acid reactive substances (TBARS) was performed to assess lipid peroxidation (54) (Bioassays Systems, Hayward, CA). Endogenous antioxidant activity, assessed by superoxide dismutase (SOD) and catalase (CAT) activity, were assayed in the plasma (51) (Cayman Chemical Company, Ann Arbor, MI) as well as ascorbic acid levels (3) (CosmoBio, Carlsbad, CA). A lipid panel and complete blood count were assessed by standard clinical techniques.

Statistical Analyses

Statistical analyses were performed using commercially available software (SPSS 17.0, Chicago, IL). A repeated measures analysis of variance 2x5 (ANOVA) ($\alpha < 0.05$) was used to determine if the oxidative stress/antioxidant assays and vascular responses to placebo and antioxidant supplementation for FMD and RH differed between healthy controls, CHF patients, and the HTx groups. Tukey's Honestly Significant Difference test

was conducted to evaluate pairwise differences among the means. A one-way ANOVA was used to determine differences in subject characteristics. All data are expressed as means ± standard error (SE).

Results

Subject Characteristics

The healthy controls, CHF patients, and HTx recipients were well matched for age and most other physical characteristics (Table 3). Other than disease state and subsequent medications (Table 4) there were only significant differences between groups in terms of blood glucose, low-density lipoprotein (LDL), hemoglobin, hematocrit, and red blood cells, however, most values fell within the normal range (Table 3).

Flow-mediated Dilation (FMD)

Baseline vascular function, as measured by PL FMD, was not different between the healthy age-matched controls (6.8 ± 1.9 %), recent, < 3 yrs post-HTx group (8.1 ± 1.2 %), and the 5-10 yrs post-HTx group (5.5 ± 1.0%) (Figure 5). In contrast, PL FMD was lower in the CHF patients (4.5 ± 0.7%) and in the > 14 yrs post-HTx recipients (2.9 ± 0.8%) compared to the controls. There was a significant negative correlation between time post-HTx and PL FMD ($r = -0.52$) (Figure 6). The antioxidant intervention increased FMD by 35% in the controls (PL 6.8 ± 1.9%; AOC 9.2 ± 1.0%) and by 55% in the > 14 yrs post-HTx recipients (PL 2.9 ± 0.8%; AOC 4.5 ± 1.3%). The AOC had no measurable vascular effect in any of the other patient groups. Shear rate was not different between groups and therefore was not utilized to normalize FMD was not normalized for shear rate.

Resting Blood Flow and Reactive Hyperemia (RH)

Resting blood flow did not differ between healthy controls, CHF, and HTx recipients (Figure 7A). Similarly, RH, both in terms of peak (Control 742 ± 77 ml/min, CHF 707 ± 104 ml/min, < 3 yrs post-HTx 845 ± 97 ml/min, 5-10 yrs-post HTx 858 ± 93 ml/min, and > 14 yrs post-HTx 936 ± 170 ml/min) and AUC (Control 561 ± 65 ml, CHF 625 ± 87 ml, < 3 yrs post-HTx 673 ± 82 ml, 5-10 yrs post-HTx 728 ± 82 ml, and > 14 yrs post-HTx 589 ± 71 ml) , was not different between groups. There was no effect of the AOC on peak RH (Control 724 ± 89 ml/min, CHF 705 ± 98 ml/min, < 3 yrs post-HTx 781 ± 88 ml/min, 5-10 yrs post-HTx 796 ± 92 ml/min, and > 14 yrs post-HTx 760 ± 72 ml/min) or AUC (Control 549 ± 85 ml, CHF 578 ± 83 ml/min, < 3yrs post-HTx 651 ± 75 ml, 5-10 yrs-post HTx 696 ± 115 ml, and > 14 yrs post-HTx 548 ± 62 ml) (Figure 7B).

Oxidative Stress and Antioxidant Capacity

Baseline (PL) plasma ascorbate levels were significantly higher in the controls compared to all of the patient groups (Figure 8A). The AOC significantly elevated plasma ascorbate concentrations in all groups 2 hrs after the ingestion of the first dose (Control 16.3 ± 2.1 to 26.9 ± 1.8 ug/ml; CHF 13.7 ± 0.7 to 20.7 ± 1.2 ug/ml; < 3 yrs post-HTx 12.9 ± 1.0 to 19.4 ± 4.7 ug/ml; 5-10 yrs post-HTx 11.7 ± 1.2 to 15.6 ± 1.7 ug/ml; > 14 yrs post-HTx 12.5 ± 0.6 to 16.3 ± 1.2 ug/ml), although all the patient groups still exhibited significantly lower levels than the controls (Figure 8A). TBARS did not significantly differ between groups (Figure 8B). However, with the exception of the patients with CHF all groups exhibited a tendency for a reduction in TBARS following AOC ingestion with the < 3 yrs post-HTx and > 14 yrs post-HTx groups nearing

significance ($p = 0.07$ and $p = 0.06$, respectively). Measures of SOD and CAT were not significantly affected by the ingestion of the AOC and there were no differences between groups.

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Discussion

This study sought to determine the changes in vascular function and the role of oxidative stress from health to the development of CHF, HTx, and beyond. Utilizing FMD to assess endothelium-dependent vascular function across the continuum, we documented reduced vasodilatory capacity in CHF patients, which was improved or normalized in early HTx recipients, and then an eventual decline in vascular function in the HTx recipients that were the furthest time from transplantation (> 14 yrs post-HTx) to a level that was comparable to, if not worse than, pretransplantation (CHF). Interestingly, unlike the other patient groups, the acute ingestion of the AOC was able to significantly increase FMD by 55% in these > 14 yrs post-HTx recipients suggesting that free radicals, and the associated decrease in NO bioavailability, are largely responsible for their endothelial dysfunction. Somewhat surprisingly, RH, an index of microvascular function, was not different across the groups and there was no effect of the AOC, highlighting the differing physiology/pathophysiology assessed by FMD and RH. Also of significant importance to the interpretation of these data is the fact that these observations across time (e.g., time post-HTx) were not confounded by aging as the controls and all patient groups were of similar age. These findings not only highlight the transient nature of vascular function over the course of CHF development and following HTx but also reveal the significant deterioration in endothelium-dependent vasodilation

in HTx recipients who are one to two decades beyond surgery. This ultimate decline, as with the controls, appears to be a consequence of a free radically-mediated reduction in NO bioavailability.

Endothelial Function in CHF and HTx Recipients

It is well accepted that peripheral endothelial function is impaired in patients with CHF (10, 23), largely due to the reduction in shear stress as a consequence of an attenuated cardiac output, reduced levels of physical activity, elevated peripheral vasoconstriction, and neurohormonal activation. Data from the current study are in agreement with this dogma as FMD, a reliable noninvasive measurement of endothelial function, was significantly blunted in CHF patients compared to the healthy, age-matched controls (Figure 5). Interestingly, vascular function appears to normalize rapidly following HTx and remain at this level for an extended period of time, as both the early (< 3 yrs post-HTx) and middle transplant (5-10 yrs post-HTx) recipients, exhibited a similar vasodilatory capacity to that of the controls. Both Kubo et al. (26) and Roig et al. (41) documented a normalization of endothelial function within the first year following HTx, which is most likely a result of an improved cardiac output with the donor heart in place and a reduction in the elevated sympathetic stimulation common in CHF (28). However, the endothelial function in HTx recipients as a whole is still somewhat controversial (37, 43).

To our knowledge, this was the first study to include healthy age-matched controls, CHF patients, and a comprehensive cross-sectional group of HTx recipients at three distinct time points following transplantation, particularly a group that was > 14 yrs

post-HTx and actually averaging 19 yrs after surgery. With this approach, we were able to determine that although endothelial-dependent vasodilation, as measured by FMD, improves soon after HTx, vascular function eventually declines to a level similar to that of CHF patients. Thus, despite having normalized central hemodynamics as a consequence of the donor heart, vascular function progressively declines as time passes following HTx (Figure 6) to a level comparable to pre-HTx (e.g., CHF) and is likely not a function of age-related changes, as age was very similar across all groups. Heart transplantation is a multifaceted intervention that not only normalizes central hemodynamics, but also requires a very invasive surgical procedure and the use of a foreign organ that comes with its own complications. Therefore, there are a multitude of factors that may contribute to endothelial dysfunction after HTx, including cytomegalovirus infection (38), exposure to cold ischemia (14), preservation solutions (40), reperfusion at the time of implantation (35), and, perhaps most significant and germane to those living for more than one to two decades post HTx, are chronic immunosuppression and complex long-term pharmacological therapy (22).

Cyclosporine is one of the original, most commonly used, immunosuppressant for heart transplant recipients. Prior research has revealed that cyclosporine may result in endothelial dysfunction (22), as it has direct cytotoxic effects on the endothelium (55), impairs endothelium-derived relaxing factor release (48), and increases endothelin production (4). Cyclosporine has also been linked to increased sympathetic tone, new-onset hypertension, and impaired peripheral blood flow in HTx recipients (44) and this may, at least in part, be mediated by free radicals (7). Our data provide further support for the idea that long-term cyclosporine exposure contributes to endothelial dysfunction

as 75% of the > 14 yrs post-HTx recipients were currently on cyclosporine (Table 4). Although there is not as much evidence as there is for cyclosporine, some of the newer immunosuppressants, such as tacrolimus, rapamycin, and mycophenolate mofetil, also appear to induce dysfunction of the vasculature (22, 50). Future research is needed to determine the long-term vascular effects of patients who have predominantly received these later generation immunosuppression therapies compared to the patients using cyclosporine. In addition to immunosuppressants, corticosteroids, another crucial part of the pharmacological regimen of HTx recipients, have been associated with dyslipidemia and glucose intolerance in the transplant patients (29, 47), potentially also contributing to the apparent deterioration in vascular function following transplantation.

Reactive Hyperemia in CHF and HTx Recipients

In contrast to FMD, the RH response represents both endothelium-dependent and independent vasodilation of the microvasculature (30, 31), but is also inversely related to cardiovascular disease risk factors (32). We hypothesized that RH would follow a similar pattern to the FMD measurements, such that when compared to controls, RH would be reduced in CHF patients, improve immediately following HTx, and progressively decline thereafter. In fact, resting limb blood flow and reactive hyperemia, both in terms of peak, and AUC were not different between groups (Figure 7). Thus, there was no evidence of microvascular dysfunction in the controls, CHF patients, or HTx recipients, which is at odds with recently published data. Indeed, Mitchell et al. (32) with data from the Framingham Heart Study, revealed that RH was more tightly correlated to cardiovascular risk factors than FMD so it seems that this would be reflected in the RH response in the

present study. However, the body of literature related to endothelial-independent vascular function in CHF patients is quite equivocal (15, 25, 34) and there is convincing evidence that endothelial-independent vascular function is not attenuated in HTx recipients (26, 41). Therefore, we speculate that our normal RH response was largely influenced by endothelium-independent vasodilation which resulted in a less sensitive measurement, as compared to FMD, which would explain our lack of differences in any of the groups in either PL or AOC conditions. In support of this apparent dichotomy, in peripheral artery disease patients, Huang et al. (21) found both FMD and RH to be significant predictors of cardiovascular events, but, of the two methods, FMD proved to be the stronger predictor of cardiovascular risk which could also potentially be attributed to the recent improvement and standardization in the FMD technique (16). It should also be noted that microvascular function does not necessarily equate to conduit vessel endothelial function which would also explain our divergent results with the FMD and RH tests.

Free Radicals and Vascular Function in CHF and HTx Recipients

It has been suggested that free radicals reduce vasodilation and limb blood flow by limiting NO bioavailability in the healthy older population (8), in CHF patients (1, 19), and HTx recipients, especially those receiving cyclosporine therapy (7). Prior studies suggest that antioxidant supplementation can restore endothelial function in healthy older individuals (8, 32), and this is supported by the current study which revealed a significant 34% increase in FMD in the healthy controls following ingestion of the AOC. It is well accepted that heart failure is characterized by low NO bioavailability

stemming from a combination of an attenuated L-arginine-nitric oxide pathway (24), reduced NO-synthase gene expression (46), a reduction of mechanical shear induced stimuli, and increased free radical formation as evidenced by increased plasma lipid peroxides (2). Previous research by Hornig et al. (18) suggests that endothelial function was improved in CHF patients following Vitamin C administered intra-arterially and following 4 weeks of oral therapy. These researchers were also able to attribute the beneficial effect of the Vitamin C to be NO mediated by the use of NO antagonist N^G-monomethyl-L-arginine which attenuated the effect of the Vitamin C supplementation. Therefore, we hypothesized that the ingestion of an acute AOC would attenuate the circulating levels of free radicals and improve vascular function with more pronounced effects in the CHF patients and the HTx recipients with a longer time post-surgery. As illustrated in Figures 5 and 7, there was no difference in vascular function, as assessed by FMD and RH, following the acute ingestion of AOC in the CHF patients, the early (<3 yrs post), or middle (5-10 yrs post) HTx recipients, which is not all that surprising considering the highly complex and varied pharmacological therapies of these patients, the low acute dosage of antioxidants given, and the relatively normal FMD responses seen in these two groups of HTx recipients. However, in the HTx recipients furthest time after surgery (> 14 yrs post), who had a significantly lower FMD and the longest immunosuppressant use, the AOC resulted in a significant 55% increase in FMD (Figure 5), implying that this attenuation in vascular function can largely be corrected by decreasing free radical concentration and thereby increasing NO bioavailability.

Ingestion of the AOC resulted in a significant increase in plasma vitamin C levels (Figure 8A) in the healthy controls, CHF patients, and all the HTx recipient groups.

Thus, the AOC was able to increase circulating antioxidant capacity in all of these individuals. There was no significant change in TBARS that serves as a "footprint" of total oxidative stress, following ingestion of the AOC (Figure 8C). However, there was an overall trend for TBARS to decrease in the controls and all the HTx recipients but it is likely that the sensitivity of this assay was not adequate to detect acute changes, if any, in oxidative stress. Such TBAR results are supported by Silvestro et al. (45) who reported that in patients with intermittent claudication, there was no relationship between TBARS and FMD following vitamin C infusion and suggested that TBARS is unable to reflect acute changes occurring within the vasculature.

Our group has previously demonstrated that in healthy older individuals, the AOC used here can effectively increase NO bioavailability to a level that can acutely improve endothelium-dependent vasodilation (8). This is supported in our control group by the elevated plasma ascorbate levels (Figure 8A) and the resultant significant increase in FMD, which is generally thought of as a bioassay of NO bioavailability (9). However, the pro- and antioxidant balance in the CHF patients and HTx recipients is likely much more complicated. Ellis et al. (11) reported oxidative stress, as measured by TBARS, to be higher in CHF patients compared to controls, but within the CHF patients this response was more exaggerated in those patients with an ischemic versus non-ischemic etiology. The current study had an approximately equal distribution of ischemic and non-ischemic CHF patients (Table 4) which may also contribute to the lack of effect our AOC had on the TBARS analyses. Additionally, the study by Ellis et al. (11) reported that only long-term (1 month) Vitamin C supplementation had a significant effect on TBARS, whereas a short-term arterial infusion of Vitamin C at a much greater concentration had

no effect, again suggesting that this assay may not be appropriate for measuring acute changes.

Clinical Perspective

CHF is a major cause of morbidity and mortality in the United States and according to the American Heart Association is the leading cause of hospitalization among those individuals on Medicare (42). In recent decades heart transplantation has advanced from an experimental procedure to an accepted life-extending therapy for patients with the most advanced stages of heart failure. Therefore, as quality of life and survival rates continue to climb for HTx patients, there is greater interest in understanding the long term effects of HTx, particularly in terms of vascular function as this appears to be a good predictor of future cardiovascular disease (27). The present study suggests that although vascular function is initially normalized following HTx, long-term immunosuppressant therapy may have extremely deleterious free-radically-mediated effects on endothelial-dependent vasodilation. The documentation of declining vascular function as time progresses in the present study has potentially great clinical importance in the realm of cardiovascular risk and increasing quality of life in long term HTx recipients.

Conclusion

In conclusion, this study has documented that endothelial-dependent vasodilation, determined by FMD, is reduced in CHF patients, and with a comprehensive, cross-sectional approach has revealed a normalization of vascular function following HTx

which gradually declines to a level similar to CHF patients in the years following surgery. Interestingly, the attenuated vascular function in HTx recipients one to two decades following surgery is most likely related to decreased NO bioavailability, as an acute dosage of oral antioxidants and a likely decrease in free radicals significantly improves their FMD.

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Disclosures

There are no conflicts of interest to report.

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Table 3. Subject characteristics

	Controls N = 12	HF N = 14	HTx (< 3 yrs) N = 12	HTx (5-10 yrs) N = 12	HTx (>14 yrs) N = 11
Male/Female	10/2	13/1	11/1	10/2	9/2
Age (yrs)	58 ± 4	62 ± 2	58 ± 3	58 ± 4	67 ± 2
Weight (kg)	94 ± 4	97 ± 5	94 ± 9	93 ± 5	78 ± 5
Height (cm)	176 ± 3	177 ± 2	174 ± 4	180 ± 2	172 ± 2
Body mass index (kg/m^2)	26 ± 1	31 ± 1	34 ± 7	29 ± 1	26 ± 1
Systolic blood pressure (mmHg)	127 ± 4	114 ± 2	124 ± 5	125 ± 3	120 ± 6
Diastolic blood pressure (mmHg)	79 ± 2	72 ± 2	77 ± 4	83 ± 3	73 ± 5
Glucose (mg/dL)	94 ± 4	137 ± 20 *	120 ± 15	93 ± 5 †	113 ± 14
Cholesterol (mg/dL)	175 ± 14	149 ± 12	146 ± 12	138 ±11	143 ± 6
HDL (mg/dL)	50 ± 4	39 ± 2	40 ± 2	42 ± 3	46 ± 5
LDL (mg/dL)	113 ± 13	91 ± 9	91 ± 10	75 ± 9 *	76 ± 5 *
Triglycerides (mg/dL)	101 ± 19	134 ± 18	116 ± 13	129 ± 21	154 ± 20
Hemoglobin (g/dL)	15 ± 0.6	15 ± 0.5	13 ± 0.4 *†‡	15 ± 0.4	13 ± 0.3 *‡
Hematocrit (%)	45 ± 1.6	44 ± 1.6	39 ± 1.6 *‡	45 ± 1.2	40 ± 0.8 ‡
RBC (M/ uL)	5.1 ± 0.2	4.8 ± 0.2	4.3 ± 0.2 *‡	5.1 ± 0.2	4.3 ± 0.1 *‡
WBC (K/uL)	5.3 ± 0.4	7.5 ± 0.6	6.1 ± 0.7	6.5 ± 0.6	5.5 ± 0.5

Mean ± standard error; HDL, high density lipoprotein; LDL, low density lipoprotein; RBC, red blood cells; WBC, white blood cells.
(*) Significantly different from control; (†) Significantly different from HF; (‡) Significantly different from HTx (5-10 yrs).

Table 4. Characteristics pertinent to the CHF and HTx recipient groups

	HF N = 14	HTx (< 3 yrs) N = 12	HTx (5-10 yrs) N = 12	HTx (> 14 yrs) N = 11
Diagnosis (ischemic cardiomyopathy)	8/14	7/12	6/12	6/11
Diagnosis (non-ischemic cardiomyopathy)	6/14	5/12	6/12	5/11
Time post-HTx (months ± SE)	NA	13 ± 4 (≈ 1 yr)	85 ± 7 (≈ 7 yrs)	226 ± 12 (≈ 19 yrs)
History of Rejection (# of all cases)	NA	1/12	1/12	2/12
Left Ventricular Ejection fraction (%)	29 ± 4	66 ± 2*	61 ± 2*	61 ± 2*
Diabetic (# of all cases)	6/14	7/12	3/12	6/11
Medications:				
Cyclosporine (# of all cases)	0/14	2/12	4/12	8/11
Tacrolimus (# of all cases)	0/14	10/12	7/12	2/11
Azathioprine (# of all cases)	0/14	2/12	2/12	0/11
Mycophenolic acid (# of all cases)	0/14	9/12	7/12	5/11
Sirolimus (# of all cases)	0/14	0/12	4/12	2/11
Prednisone (# of all cases)	0/14	6/12	2/12	4/11
Beta-blocker (# of all cases)	14/14	1/12	4/12	6/11
ACE-Inhibitor (# of all cases)	10/14	6/12	5/12	2/11
Angiotensin Receptor Blocker (# of all cases)	3/14	2/12	4/12	2/11
Statin (# of all cases)	11/14	10/12	9/12	5/11
Diuretic (# of all cases)	9/14	3/12	5/12	3/11
Calcium channel-blocker (# of all cases)	1/14	5/12	3/12	4/11
Mean ± SE; (*) Significantly different from HF				

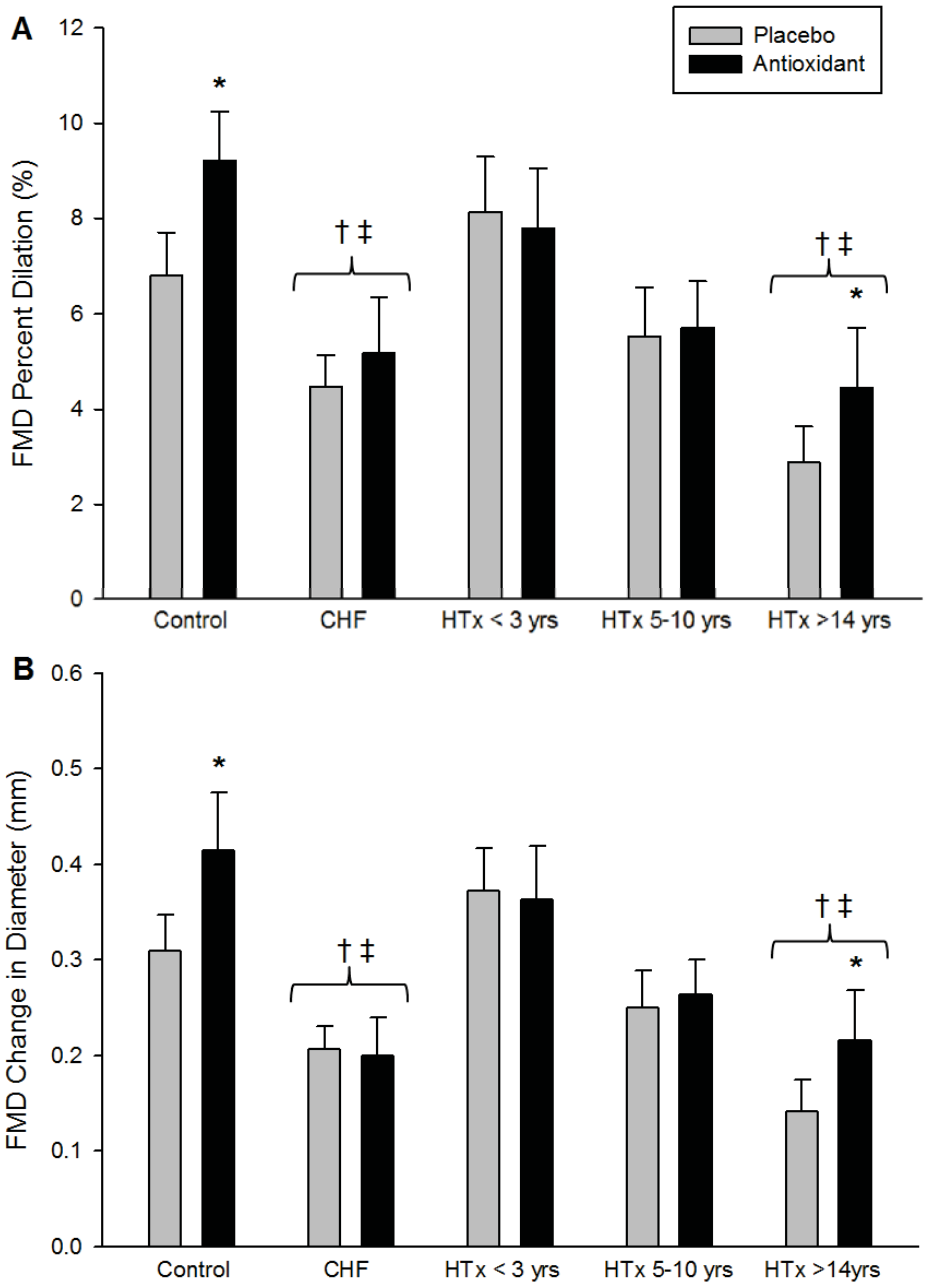

Figure 5. FMD measurements in healthy controls, CHF patients, and three groups of HTx recipients. **(A)** Brachial artery FMD following 5 min cuff occlusion expressed as a percent change from pre-cuff baseline in healthy controls, CHF patients, and three groups of HTx recipients. (*) Significantly different from Placebo; (†) Significantly different from Controls; (‡) Significantly different from HTx (< 3 yrs). **(B)** Brachial artery FMD expressed as absolute change in diameter from pre-cuff baseline in healthy controls, CHF patients, and 3 groups of HTx recipients. (*) Significantly different from Placebo; (†) Significantly different from Controls; (‡) Significantly different from HTx (< 3 yrs). Values are means ± SE.

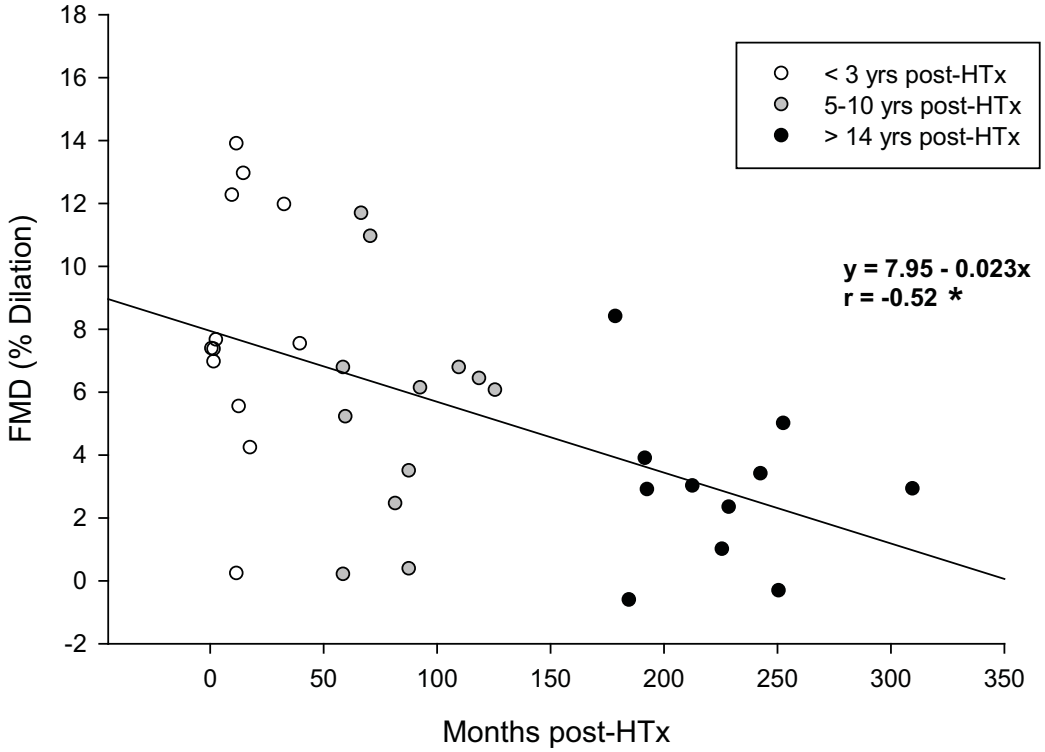

Figure 6. Time post-HTx and its influence on FMD (% diameter change).

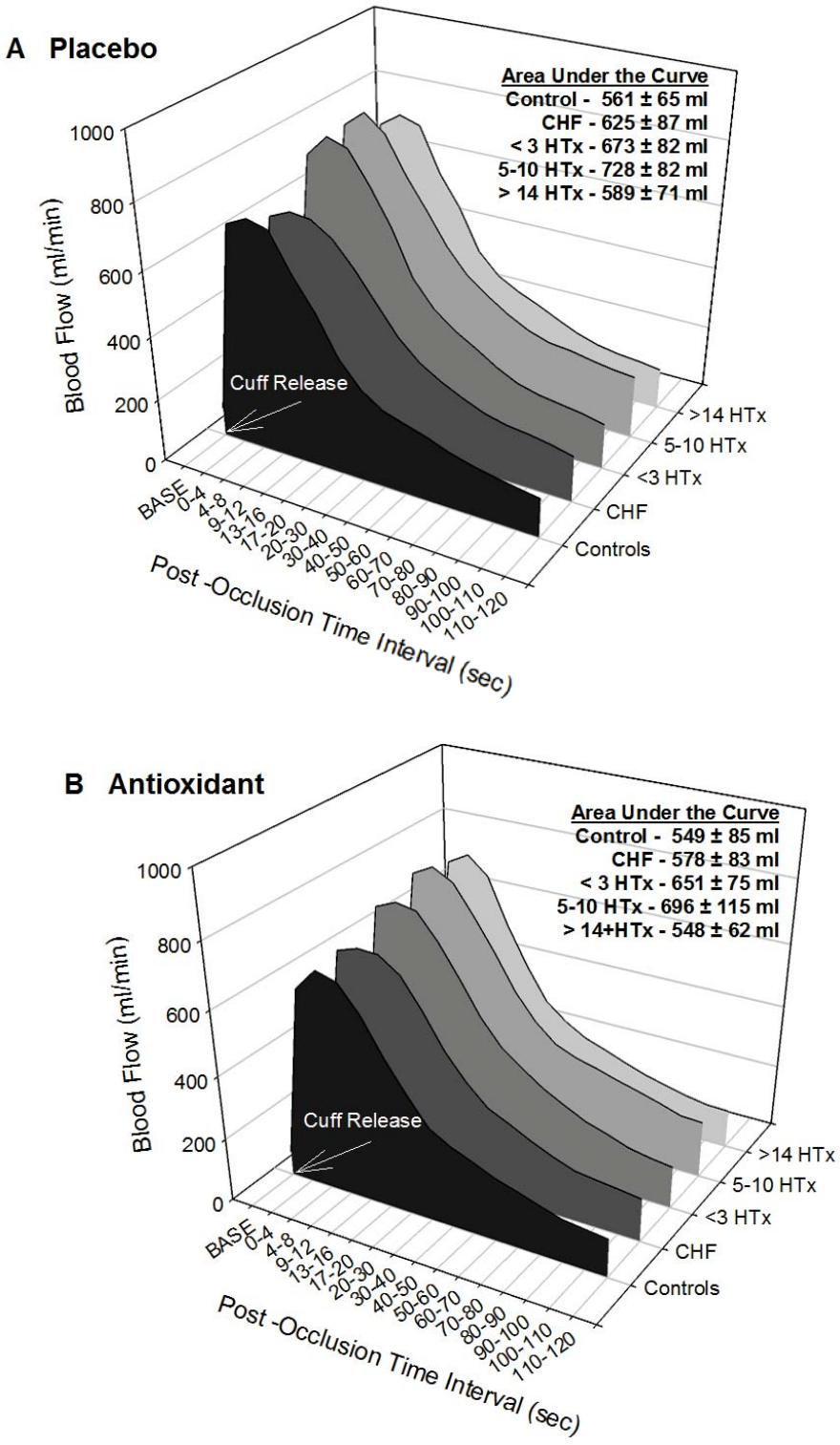

Figure 7. Resting brachial artery blood flow and RH following cuff occlusion in healthy controls, CHF patients, and three groups of HTx recipients. There were no significant differences at baseline, peak RH, or AUC for either Placebo **(A)** or Antioxidant **(B)**.

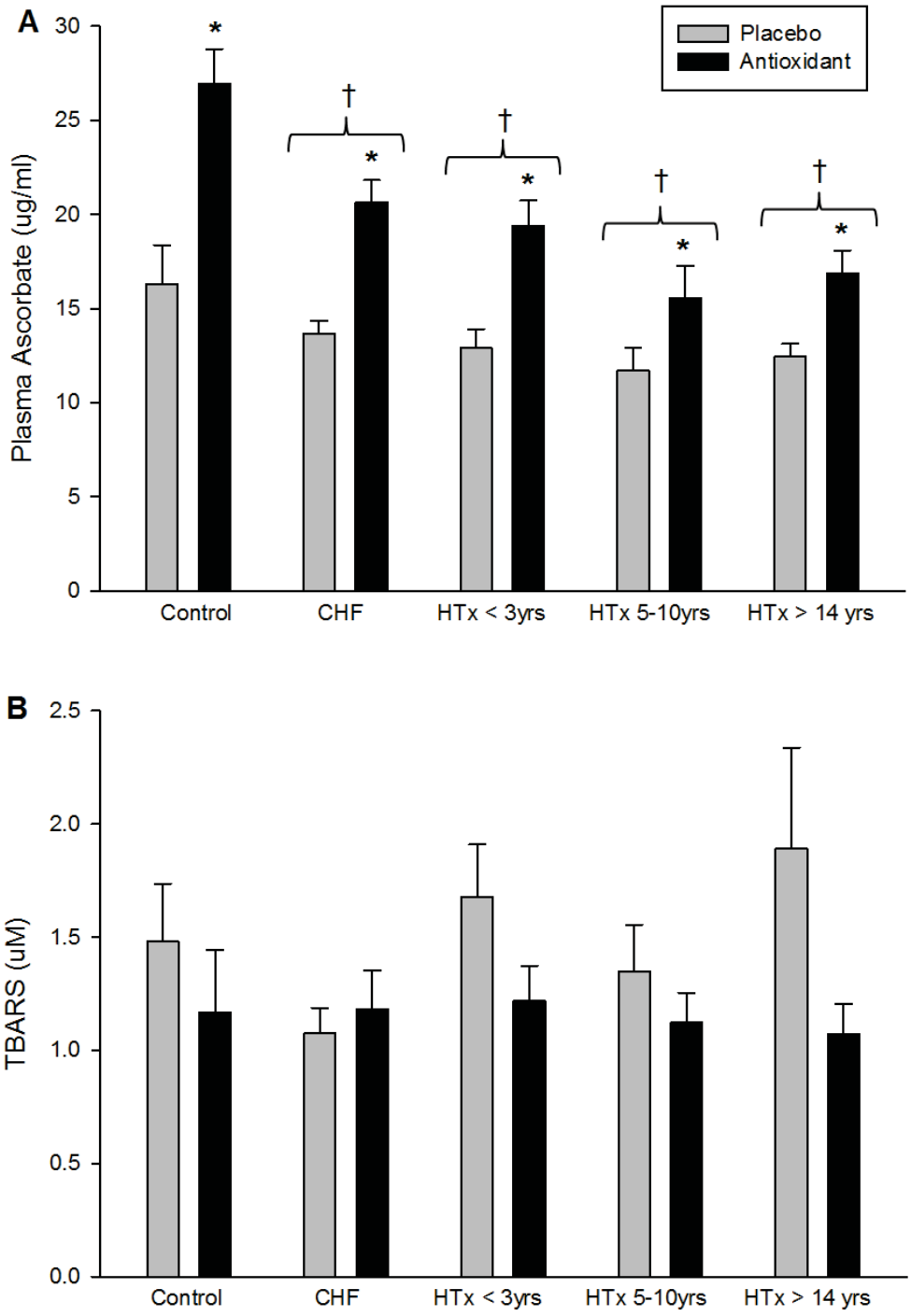

Figure 8. Quantitative assessment of antioxidant efficacy in healthy controls, CHF patients, and three groups of HTx recipients. **(A)** plasma ascorbate; **(B)** Thiobartituric Acid Reactive Substances (TBARS). Values are means ± SE. (*) Significantly different from Controls; (†) Significantly different from Placebo.

CHAPTER 4

EVIDENCE OF FREE RADICAL-MEDIATED SYSTEMIC VASCULAR RESISTANCE IN PATIENTS WITH CHRONIC HEART FAILURE

Abstract

To better elucidate the link between free radicals and hemodynamic control in patients with chronic heart failure (CHF), we studied 10 patients and 10 age matched healthy controls at rest and during handgrip exercise with either an acute oral antioxidant cocktail (AOC (Vitamin C, E, and α lipoic acid)) or placebo (PL). To assess central and peripheral hemodynamics, mean arterial pressure (MAP), cardiac output (CO), systemic vascular resistance, brachial artery blood flow, and peripheral (arm) vascular resistance were determined. Compared to controls, patients with CHF exhibited greater oxidative stress, measured by thiobarbituric acid reactive substances (TBARS) (~40%), and evidence of endogenous antioxidant compensation, measured by superoxide dismutase (SOD) activity (~45%). The AOC increased plasma ascorbate by ~32% in both the CHF patients and controls. The ingestion of the AOC had significant systemic hemodynamic effects which were only evident in the patients with CHF, both at rest and throughout exercise. Specifically, the AOC further reduced the patients already lower MAP (~5%), increased CO (~10%), and caused a fall in systemic vascular resistance (~12%). In contrast, peripherally, brachial artery blood flow tended to be reduced, and peripheral (arm) vascular resistance was unchanged. Based upon the recognized link between free radicals and sympathetic nerve activity in patients with CHF, these data imply that systemic vascular resistance appears, at least in part, to be free radically-mediated; however, this finding does not appear to be the direct result of muscle-specific changes in peripheral vascular resistance.

Introduction

In patients with chronic heart failure (CHF) exercise capacity and activities of daily living are often limited, resulting in decreased physical capacity and greater morbidity and mortality. In these patients it is often assumed that exercise capacity depends on the severity of cardiac function (43, 50), but ejection fraction and measures of left ventricular performance do not always reflect exercise tolerance (16, 17). These observations have promoted the concept that the limited exercise capacity in CHF patients may also result from a complicated pathophysiology in the periphery that includes alterations in skeletal muscle (12), endothelial function (11, 19), and augmented sympathoexcitation (7). Previous research has established that in CHF animal models (41) as well as CHF patients (30, 39), sympathetic nerve activity is not only higher at rest but even more exaggerated during exercise. This peripheral neural reflex, known as the exercise-pressor reflex, contributes to an increase in blood pressure, heart rate (HR), and peripheral vasoconstriction during exercise (40) and is thought to play an integral role in the regulation of blood flow to metabolically active and inactive vascular beds. While the increased sympathetic nerve activity in CHF is compensatory in nature it is most likely detrimental to exercise capacity (29), with the central and peripheral hemodynamic impact remaining unclear (15, 25, 55).

In vivo, oxidative stress represents an imbalance between free radical production and endogenous antioxidant defenses and is often associated with the onset and development of CHF (3, 20, 27). Elevated free radicals, particularly superoxide, have been linked to peripheral hypoperfusion, peripheral endothelial dysfunction, and exaggerated sympathetic nerve activity in the CHF patient population (22, 44, 46), which

are all likely contributors to exercise intolerance. Prior research, in an animal model of CHF, has revealed that decreasing free radicals by an arterial antioxidant infusion attenuated sympathetic nerve activity and MAP (22). Interestingly, in contrast to the decrement in central parameters, indices of oxidative stress have been closely correlated with peak exercise oxygen consumption and New York Heart Association (NYHA) functional classification of CHF patients (20, 28). However, the contribution of elevated levels of free radicals to hemodynamic control in patients with CHF at rest and during exercise is largely unknown and remains to be elucidated.

Consequently, using rhythmic isometric handgrip exercise and an acute oral AOC, this study sought to better characterize the role of free radicals in the regulation of central and peripheral hemodynamics at rest and during exercise in patients with CHF. Specifically, we hypothesized that, when compared to healthy age-matched controls, patients with CHF will exhibit 1) basal evidence of elevated oxidative stress, and that during handgrip exercise the patients will reveal, 2) exaggerated central hemodynamic responses, particularly in terms of HR and MAP, 3) a significant AOC-induced reduction in systemic vascular resistance that will attenuate the increase in MAP, and 4) a similar AOC-induced interplay between MAP and peripheral (arm) vascular resistance, such that peripheral vascular resistance will be significantly reduced.

Methods

Subjects

A total of 20 subjects (10 healthy controls and 10 NYHA Class II and III CHF patients), were recruited either by flier or in the CHF clinics at the University of Utah and the Salt Lake City VA Medical Center. The protocol was approved by these institutions and written informed consent was obtained. Subject characteristics and the pharmacological therapy of the CHF patients at the time of the study are reported in Tables 5 and 6. All studies were performed in a thermoneutral environment at least three days apart to allow for washout of the oral AOC. Subjects reported to the laboratory in a fasted state, and without caffeine or alcohol use for 12 and 24 hrs, respectively. Additionally, subjects had not performed any exercise within the past 24 hrs and if antioxidants and/or a multivitamin were part of a subject's daily routine they were asked to refrain for at least 3 days prior to testing.

Handgrip Exercise Protocol

Subjects were instrumented with a Finometer (Finapres Medical Systems, Amsterdam, The Netherlands) to assess central hemodynamics and then rested supine for approximately 20 min prior to baseline resting measurements of MAP, HR, SV, and CO. Ultrasound Doppler was used to assess resting brachial artery diameter and blood velocity. While lying in a supine position with the arm resting at 90°of abduction, with the elbow joint extended at heart level, subjects performed a maximal voluntary contraction (MVC) (average of three maximal efforts). Following sufficient rest, dynamic handgrip exercise (1 Hz) was performed at three workloads (4-8-12 kg) using a

commercially available handgrip dynamometer (TSD121C, Biopack Systems Inc., Goleta, CA). The subjects squeezed the dynamometer to the sound (60 beats/min) of a digital metronome (Seiko DM70, CA). A computer monitor was positioned so that the subjects could observe their force output and make corrections whenever necessary. Each exercise-stage lasted 3 min to ensure the attainment of steady-state hemodynamics. Central hemodynamics, brachial artery diameter, and blood velocity were assessed continuously, but only the steady-state data (last 60 sec) attained during each exercise-stage were analyzed.

Central Hemodynamic Assessments

Throughout the entire protocol HR, SV, CO, and MAP signals from the Finometer (Finapres Medical Systems BV, Amsterdam, The Netherlands) underwent A/D conversion and were simultaneously acquired (200 Hz) using commercially available data acquisition software (AcqKnowledge, Biopac Systems). SV was calculated using the Modelflow method which includes age, gender, height and weight in its algorithm (Beatscope version 1.1; Finapres Medical Systems BV, Amsterdam, The Netherlands) (5), and has been documented to accurately track CO during a variety of experimental protocols including exercise (8, 9, 14, 42, 47). CO was then calculated as the product of HR and SV. Systemic vascular resistance was calculated as MAP/CO.

Brachial Artery Assessments

Brachial artery diameter and blood velocities were measured with ultrasound Doppler using a General Electric (GE) Logiq 7 ultrasound system (GE Medical Systems,

Milwaukee, WI, USA). This system was equipped with a linear array transducer operating at an imaging frequency of 12-14 MHz, with frequency selected to optimize image quality according to vessel depth (18). Blood velocity was obtained with the same transducer with a Doppler frequency of 5 MHz. An insonation angle of 60º or less was utilized and the sample volume was centered within the vessel and maximized according to vessel size. Brachial artery diameters were then analyzed off-line using commercially available software (Medical Imaging Applications, LLC, Coralville, IA). Angle-corrected and intensity-weighted mean velocities (V_{mean}) were determined using commercially available software (Logic 7). Using V_{mean} and arterial diameter, brachial artery blood flow was calculated as: Blood flow = $V_{mean}\pi$ (arterial diameter/2)^2 x 60, where blood flow is measured in milliliters per min. Peripheral (arm) vascular resistance was calculated as MAP/brachial artery blood flow.

Laminar shear stress is believed to be the mechanism that stimulates the vascular endothelium and results in subsequent vasodilation of the brachial artery (52). However, as blood viscosity was not measured, shear rate, which can be an adequate surrogate measure (35), was calculated using the following equation: Shear rate (s^{-1}) = $8 \cdot V_{mean}$ (cm/s)/arterial diameter (cm).

Antioxidant Supplementation

On two separate visits to the laboratory, separated by a minimum of 3 days, subjects received either the AOC or placebo in a balanced, single blind design. The supplements were administered 90 and 60 min prior to the FMD protocol. A split dosing was used to improve absorbance and distribution of the antioxidants. The first

antioxidant dose included Vitamin E (200 IU), Vitamin C (500 mg), Alpha-lipoic Acid (300 mg), and the subsequent dose included Vitamin E (400 IU), Vitamin C (500 mg), and Alpha-lipoic Acid (300 mg). The placebo microcrystalline cellulose capsules, that were of similar taste, color, and appearance, were also consumed in two equivalently timed doses. The efficacy of this AOC to reduce plasma free radical concentration has previously been established using ex-vivo spin trapping and electron paramagnetic resonance (EPR) spectroscopy (51).

Oxidative Stress and Antioxidant Assays

In both the placebo and antioxidant trials blood samples were obtained from the antecubital vein prior to testing (1 hr following ingestion of the second dose of either AOC or placebo). Serum and plasma samples were stored at -80°C until analysis. Lipid peroxidation, a marker of oxidative stress was assessed by quantifying thiobarbituric acid reactive substances (TBARS) (54) (Bioassays Systems, Hayward, CA). Antioxidant capacity was assessed by determining the ferric reducing ability of plasma (FRAP), using the method described by Benzie and Strain (4). Endogenous antioxidant activity, assessed by superoxide dismutase (SOD) and catalase (CAT) activity, were assayed in the plasma (49) (Cayman Chemical Company, Ann Arbor, MI) and the efficacy of the AOC to acutely raise antioxidant levels in the blood was determined by plasma ascorbic acid levels (6) (CosmoBio, Carlsbad, CA). A Lipid panel and complete blood count (CBC) were performed by standard clinical techniques.

Statistical Analyses

Ultrasound measurements were performed continuously during rest and exercise, though only the last 60 sec of each stage were analyzed, where V_{mean} was averaged across five 12 sec intervals and diameter measurements were evaluated during diastole. Statistical analyses were performed using commercially available software (SPSS 17.0, Chicago, IL). Descriptive statistics were performed on all data. A 2x2 factors (intervention and group) repeated measures analysis of variance ANOVA was used to determine if the oxidative stress/antioxidant assays and baseline hemodynamic measures differed between groups and intervention. A 2x3 factors (intervention and exercise intensity) repeated-measure ANOVA was performed to identify changes in dependent variables within groups, drug condition and across exercise intensities. Independent t-tests were used to determine differences in subject characteristics. Significant interactions were followed up with the appropriate post hoc analyses. All data are expressed as means ± standard error (SE). Statistical significance was established at $P < 0.05$.

Results

Oxidative Stress and Antioxidant Capacity

The AOC significantly elevated plasma ascorbate concentration by ~32% in both the CHF patients and controls 2 hrs after the ingestion of the first dose (CHF 14 ± 1 to 21 ± 1 ug/ml; Controls 16 ± 2 to 24 ± 1 ug/ml) (Figure 9A). The AOC also significantly elevated antioxidant capacity, as measured by FRAP, in the control group (PL 1046 ± 42 to AOC 1164 ± 41 mM/L) while the CHF patients tended to already have greater antioxidant capacity than the control group (p = 0.09) (Figure 9B). TBARS, a marker of

oxidative stress, was significantly elevated in the patients with CHF compared to the controls (Figure 9C). While the AOC lowered TBARS by ~56% in the controls (PL 0.88 ± 0.2 to AOC 0.57 ± 0.1 uM), this intervention appeared to have no such effect on the patients with CHF. Catalase activity was not different between the patients with CHF and the controls, but was increased in both groups following the AOC ingestion (Figure 9D). SOD activity was ~45% higher in the patients with CHF compared to controls and was unaltered in either group by the AOC ingestion (Figure 1E.).

Central Hemodynamics

At rest PL MAP was significantly lower in the CHF patients (86 ± 3 mmHg) compared to the controls (96 ± 3 mmHg) ($p < 0.05$) (Figure 10A), but there were no differences in HR, SV, or CO (Figure 10B). During handgrip exercise PL MAP was significantly lower in the CHF patients at all workloads compared to controls (Figure 10A). In the CHF patients, ingestion of the AOC resulted in lower MAP at rest (PL 86 ± 3 mmHg, AOC 82 ± 2 mmHg) and throughout all stages of handgrip exercise (approximately 5 mmHg at each workload) (Figure 10A). This reduction in MAP was accompanied by nonsignificant increases in HR and SV which resulted in a statistically significant increase in CO at all time points following ingestion of the AOC (Figure 10B).

Peripheral Hemodynamics

The peripheral hemodynamic responses to handgrip exercise are illustrated in Figures 10C, and E. Resting arm blood flows, measured in the brachial artery, were not

different between subject groups or treatments (PL CHF 86 ± 16 ml/min, AOC CHF 71 ± 15 ml/min; PL controls 105 ± 13 ml/min, AOC controls 99 ± 15 ml/min). During handgrip exercise brachial artery blood flow increased with increasing workload, but there was no difference in blood flow between groups under any of the conditions (Figure 10C). Similarly, there were no significant differences in vascular conductance at rest either between groups or as a consequence of ingesting the AOC (PL CHF 0.9 ± 0.1 ml/min/mmHg, AOC CHF 0.7 ± 0.1 ml/min/mmHg; PL controls 1.1 ± 0.2 ml/min/mmHg, AOC controls 1.0 ± 0.2 ml/min/mmHg) and this observation held true during exercise (Figure 10E). Brachial artery diameters were not different at rest either between groups or as a consequence of ingesting the AOC (PL CHF 5.1 ± 0.3 mm, AOC CHF 4.9 ± 0.3 mm; PL controls 4.9 ± 0.2 mm, AOC controls 5.0 ± 0.2 mm) and again this observation held true during exercise (data not shown). Shear rate increased with exercise intensity and was in most cases well related to the change in arterial diameter for both the controls ($R^2 = 0.63 \pm 0.13$) and CHF patients ($R^2 = 0.70 \pm 0.13$) (Figure 11). The AOC had no measurable effect on the relationship between shear rate and brachial artery diameter in either the patients with CHF or the controls.

Discussion

This study sought to better characterize the role of free radicals in regulating central and peripheral hemodynamics at rest and during exercise in patients with CHF using an oral AOC and dynamic handgrip exercise. Oxidative stress was confirmed to be significantly elevated in the CHF patients, but increased activity of the endogenous antioxidant SOD suggested some degree of compensation. Although the ingestion of the AOC further increased antioxidant capacity in both the patients and controls, only in the

patients with CHF did the AOC result in a significant hemodynamic response both at rest and during exercise. Specifically, in terms of central hemodynamics, the AOC resulted in a significant reduction in MAP (~5%), an increase in CO (~10%), and a fall in systemic vascular resistance (~12%). The peripheral response to the AOC contrasted starkly with these changes with arm blood flow tending to fall and peripheral (arm) vascular resistance remaining essentially unchanged. Based upon the recognized link between free radicals and sympathetic nerve activity in patients with CHF, these data provide evidence that in patients with CHF systemic vascular resistance is, at least in part, free radically-mediated. However, this does not appear to be the result of limb or skeletal muscle-specific changes in peripheral resistance. This finding has the potential to guide future investigations targeting exercise intolerance and disease progression in this population.

Oxidative Stress and Antioxidants in CHF

Oxidative stress occurs when there is an imbalance between free radical production and antioxidant defenses. Both human studies and animal models of CHF suggest that oxidative stress is elevated in this disease population and the excess free radical load has been implicated in many of the structural and functional changes that are characteristic of CHF. Elevated levels of whole-body oxidative stress in the current patients with CHF was confirmed by an elevation in TBARS, a marker of lipid peroxidation, compared to controls (Figure 9C). This is in agreement with prior work by Ellis et al. (13) who also documented elevated TBARS in patients with CHF. In terms of the endogenous antioxidant defense system in patients with CHF, previous research has been quite inconsistent with studies reporting that activity was increased (10), decreased

(23, 26), or was unchanged (28). In the present study the patients with CHF exhibited a ~45% greater SOD activity and a tendency for greater catalase activity (Figure 9D and E), but this did not achieve statistical significance. SOD is typically considered as the first line of defense against accumulating free-radicals and superoxide is the radical that is often linked to vascular dysfunction and exaggerated sympathetic nerve activity in CHF patients (22, 44). Although previous findings in terms of SOD activity in patients with CHF have been inconsistent, the current data indicating increased SOD activity can be interpreted as evidence of a compensatory upregulation due to the concomitantly greater oxidative stress evident in these patients.

The efficacy of the AOC utilized in the current study has previously been documented by the clear reduction in blood born free radicals directly measured by ex-vivo spin trapping and electron paramagnetic resonance (EPR) spectroscopy (51). Interestingly, although in the current study the AOC clearly raised antioxidant levels in both the patients with CHF and controls (Figure 9A and B), TBARS were only significantly decreased (~50%) in the healthy controls. However, this is in agreement with the findings of Ellis et al. (13) who reported that only long-term Vitamin C supplementation in CHF patients had a significant effect on TBARS, whereas short-term Vitamin C infusion had no measurable effect on this footprint of oxidative stress. Additionally of note, although there was a main effect of the AOC on the assessment of antioxidant capacity, as measured by FRAP, post hoc analyses support the qualitative assessment (Figure 9B) that this was driven by a change in the control group and not in the patients with CHF. This is likely partially explained by the tendency ($p=0.09$) for the patients with CHF to already have a greater antioxidant capacity than the controls prior to

ingesting the AOC, making the AOC-induced increase, an even smaller relative component of all the antioxidants assessed by the FRAP. Regardless, it is clear from the systemic hemodynamic data that the AOC had a clear physiological impact in the patients with CHF and not the controls and this was likely due to differences in initial levels of free radicals and oxidative stress.

Oxidative Stress and Central Hemodynamics in CHF

It is well accepted that in CHF patients, sympathetic nerve activity is augmented at rest (24, 45) and often well related to disease prognosis (2, 7). During exercise, increased group III and IV muscle afferent nerve activity results in the exercise pressor response which increases blood pressure, via sympathetic nerve activity, and ultimately blood flow to the active skeletal muscle (40). However, as sympathetic nerve activity is greater in patients with CHF than healthy individuals (30, 34) this appears to negatively impact exercise tolerance, which is likely a result of excessive peripheral vascular resistance (30, 33). Consequently, we hypothesized that in response to rhythmic isometric handgrip exercise, CHF patients would exhibit exaggerated central hemodynamic responses, particularly in terms of HR and MAP compared to the controls. This was not the case, in fact, the CHF patients in the current study had a significantly lower MAP at rest (86 ± 3 mmHg) compared to the controls (96 ± 3 mmHg) and this attenuated MAP remained evident at each exercise workload (4, 8, and 12kg) (Figure 10A). There were also no differences in resting HR, SV, or CO between the two subject groups (Figure 10). The lower MAP and unremarkable HR data in the CHF patients at rest and throughout exercise is in agreement with several prior human studies (1, 38, 39)

and can likely be attributed to maintaining the patients pharmacological therapy throughout the experiment. However, we chose this approach to both minimize patient risk and better understand the challenges these patients face in the "real world," when their disease is optimally pharmacologically controlled.

Several large clinical trials have found that decreasing sympathetic activity in CHF patients can actually improve mortality (31, 32) thus, providing an important therapeutic target, particularly during exercise when this response has the potential to be more exaggerated in CHF patients. Given the growing evidence supporting the link between oxidative stress and CHF, several studies using animal CHF models and an intra-arterial infusion of an endogenous antioxidant mimetic (e.g. mimicking the enzymatic activity of SOD) have revealed a reduction in sympathetic nerve activity and therefore the exercise pressor response during muscle contraction (22, 48). As illustrated in Figure 10 (A, B, and D), ingestion of the oral AOC by the patients with CHF in the current study resulted in significant central hemodynamic changes which were evident both at rest and across all levels of handgrip exercise: MAP was significantly reduced (~5 mmHg), slight, but nonsignificant, increases in HR and SV combined to significantly increase CO (~0.4 L/min), and systemic vascular resistance fell significantly (~2 mmHg/L/min) (Figure 10B). These findings are consistent with the reduced blood pressure and sympathetic nerve activity documented in the previously discussed CHF animal models (22, 48). While in a CHF-induced rabbit model, supplementation with beta-carotene, ascorbic acid (Vitamin C), and alpha-tocopherol (Vitamin E), a combination of antioxidants somewhat similar to those used in the current study, reduced tissue oxidative stress and attenuated the associated cardiac dysfunction, caused beta-

receptor downregulation, and reduced sympathetic nerve terminal abnormalities (37). Of importance, in the current study, we were able to reveal that systemic vascular resistance in pharmacologically-treated CHF patients is, at least in part, mediated by free-radicals. This finding provides important mechanistic insight into the exaggerated sympathetic nerve activity exhibited by patients with CHF and has the potential to guide future investigations related to exercise intolerance and disease progression in this population.

Oxidative Stress and Peripheral Hemodynamics in CHF

Previous research in CHF patients has reported that peripheral blood flow is reduced at rest (55) and during exercise (50) contributing to the exercise intolerance that is a hallmark characteristic of the disease. Although there have been considerable variations in terms of the extent and potential mechanisms responsible for the attenuated peripheral blood flow in patients with CHF due to methodological differences, exaggerated sympathoexcitation is likely a primary contributor. We hypothesized that, as with the central hemodynamics, during exercise there would be an AOC-induced reduction in peripheral (arm) vascular resistance, such that the potentially exaggerated MAP response in patients with CHF patients would not occur. However, in the placebo condition, this group of patients with CHF, using the submaximal handgrip exercise model, did not respond differently, in terms of peripheral hemodynamics, from the controls. In fact, there were no significant differences in peripheral (arm) vascular resistance or brachial artery blood flow (Figure 10C and E) at rest or during exercise between the patients and controls, with or without the AOC. Our findings, under the placebo condition, are similar to a previous study by Shoemaker et al. (38) who reported

a similarly attenuated MAP in patients with CHF and also failed to see a differences between the CHF patients and controls in terms of forearm blood flow and vascular conductance throughout handgrip exercise.

Given the AOC-induced fall in MAP and recognizing Ohms law and how it relates to the human vascular system, either arm vascular resistance, brachial artery blood flow or both would be expected to fall in this scenario. Although not statistically significant, there was such a trend in blood flow following ingestion of the AOC (Figure 10C), but there was no such trend in arm vascular resistance (Figure 10E). Thus, the impact of the AOC on peripheral hemodynamics at both rest and exercise are in stark contrast to the previously discussed central hemodynamics, revealing that the central changes do not appear to be the result of alterations in arm or skeletal muscle-specific peripheral resistance. This suggests that free radicals in patients with CHF do not impact skeletal muscle sympathetic nerve activity, but have the majority of effect on other organs.

Progressive Handgrip and Nitric Oxide-dependent Vasodilation in CHF

Although not a focus of the current study, performance of this work afforded the opportunity to examine vascular function in both controls and patients with CHF utilizing a novel approach recently championed by our group. Specifically, we have documented that as dynamic handgrip exercise elevates shear stress in an intensity-dependent manner that results in a corresponding increase in vessel diameter this model appears to provide an assessment of brachial artery vasodilatory capacity (53). Additionally, using intra-arterial N^G-monomethyl-L-arginine (L-NMMA) to block endothelial nitric oxide (NO)

synthase, we identified NO to be a major contributor to shear-induced brachial artery vasodilation during progressive handgrip exercise. Thus, progressive, dynamic handgrip exercise, as used in the current study, may be an effective tool for assessing NO bioavailability in humans. However, to date, the application of this approach to assessing vascular function has been limited to young healthy volunteers.

Therefore to compare and contrast NO mediated vasodilation in the current older controls and the patients with CHF, we examined the relationship between the shear rate developed during three stages of exercise and the change in brachial artery diameter (Figure 11). In general, both the older controls and patients with CHF support the strong linear relationship between increasing shear rate induced by incremental handgrip exercise and the resulting brachial artery vasodilation (Figure 11). Although the preponderance of data examining vascular function in patients with CHF suggests that this disease is associated with a decrement in flow mediated vasodilation (11, 19) and by inference NO bioavailability, this finding has not always been consistent (21, 36). With the current approach, there was no significant difference in the slopes of shear rate to brachial artery vasodilation relationships between the controls ($m = 0.004 \pm 0.001$) and CHF patients ($m = 0.004 \pm 0.002$). However, it is interesting to note that the slope of this relationship for both these groups was lower than the slope previously reported in young, healthy individuals ($m = 0.005 \pm 0.001$) and far greater than the slope of the young subjects with NO blockade ($m = 0.0007 \pm 0.0005$). Therefore, using this novel approach to assess NO-dependent vasodilation, a significant NO-mediated vasodilatory capacity was identified in both groups, but there was no discernible difference between the healthy controls and the patients with CHF.

Conclusion

This study has documented that both at rest and during exercise, in patients with CHF exhibiting signs of oxidative stress, central and not peripheral hemodynamics are at least partially mediated by free radicals. This finding may provide important mechanistic insight into the exaggerated sympathetic nerve activity exhibited by patients with CHF and has the potential to guide future investigations targeting exercise intolerance and disease progression in this population.

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Disclosures

There are no conflicts of interest to report.

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Table 5. Subject characteristics

	Controls	CHF
Subjects (n)	10	10
Age (yrs)	60 ± 3	62 ± 2
Weight (kg)	82 ± 3	96 ± 5 *
Height (cm)	176 ± 2	178 ± 2
Body mass index (kg/m^2)	26 ± 1	30 ± 1 *
Systolic blood pressure (mmHg)	126 ± 4	114 ± 2 *
Diastolic blood pressure (mmHg)	80 ± 2	71 ± 3 *
Glucose (mg/dL)	80 ± 5	134 ± 25
Cholesterol (mg/dL)	187 ± 9	151 ± 17
HDL (mg/dL)	48 ± 2	40 ± 3 *
LDL (mg/dL)	129 ± 9	95 ± 13 *
Triglycerides (mg/dL)	116 ± 16	109 ± 22
Hemoglobin (g/dL)	16 ± 1	15 ± 3
Hematocrit (%)	47 ± 1	43 ± 2
RBC (M/ uL)	5.4 ± 0.1	4.8 ± 0.2 *
WBC (K/uL)	5.2 ± 0.3	7.7 ± 0.7 *
Mean ± SE; HDL, high density lipoprotein; LDL, low density lipoprotein; RBC, red blood cells; WBC, white blood cells. (*) Significantly different from control.		

Table 6. Characteristics pertinent to the CHF group

	CHF
Diagnosis (ischemic cardiomyopathy)	7/10
Diagnosis (non-ischemic cardiomyopathy)	3/10
Left Ventricular Ejection fraction (%)	26 ± 3
NYHA Class II	7
NYHA Class III	3
Diabetic (# of all cases)	3/10
Medications:	
Beta-blocker (# of all cases)	9/10
ACE-Inhibitor (# of all cases)	6/10
Angiotensin Receptor Blocker (# of all cases)	2/10
Statin (# of all cases)	9/10
Diuretic (# of all cases)	7/10
Calcium channel-blocker (# of all cases)	0/10
Alpha-blocker (# of all cases)	0/10

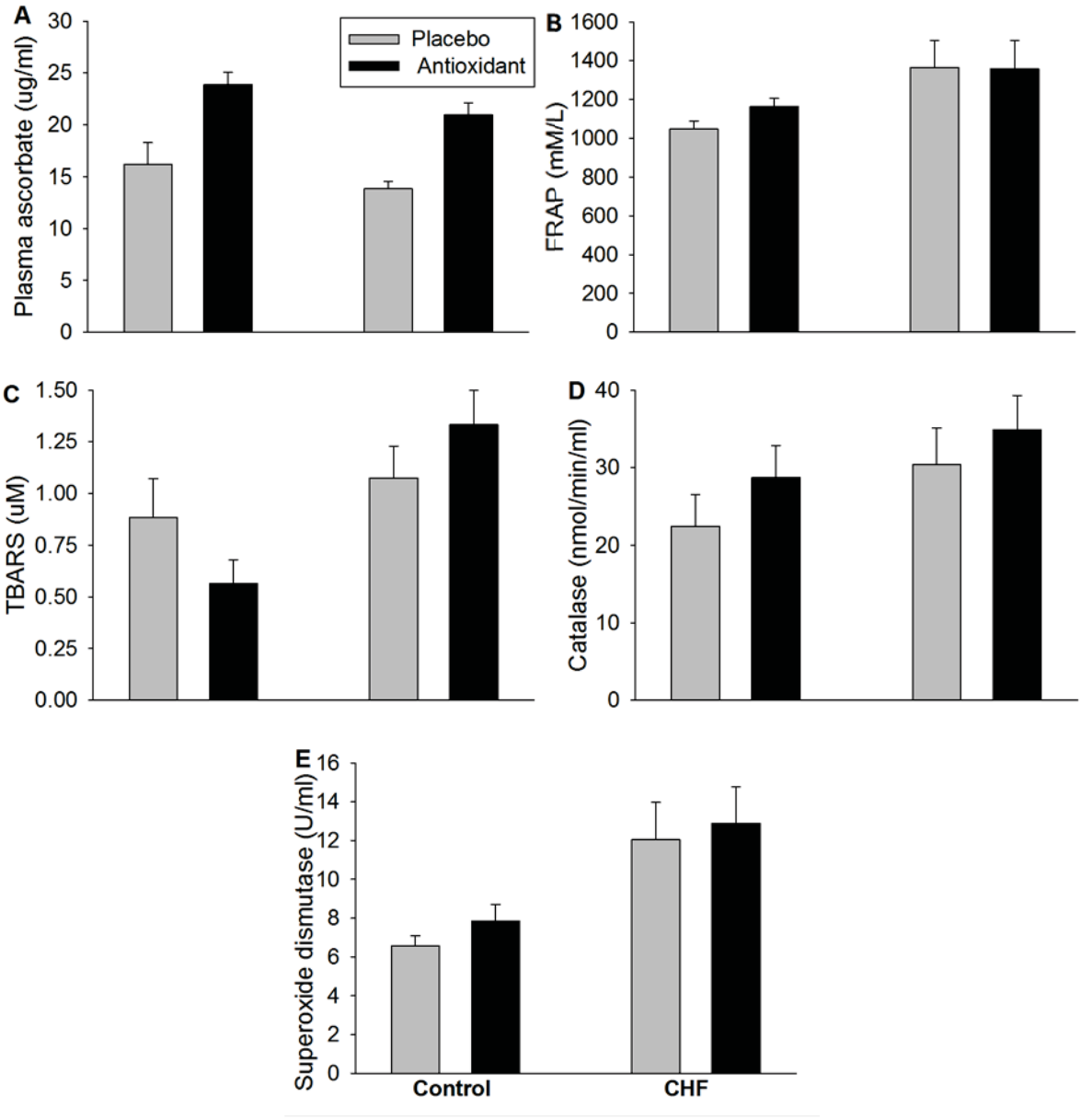

Figure 9. Quantitative assessment of oxidative stress and antioxidant markers in healthy controls and CHF patients. (A) Plasma ascorbate; (B) Ferric-reducing ability of plasma (FRAP); (C) Thiobartituric Acid Reactive Substances (TBARS); (D) Catalase; (E) Superoxide dismutase. Values are means ± SE. (*) Significantly different from Controls; (†) Significantly different from PL.

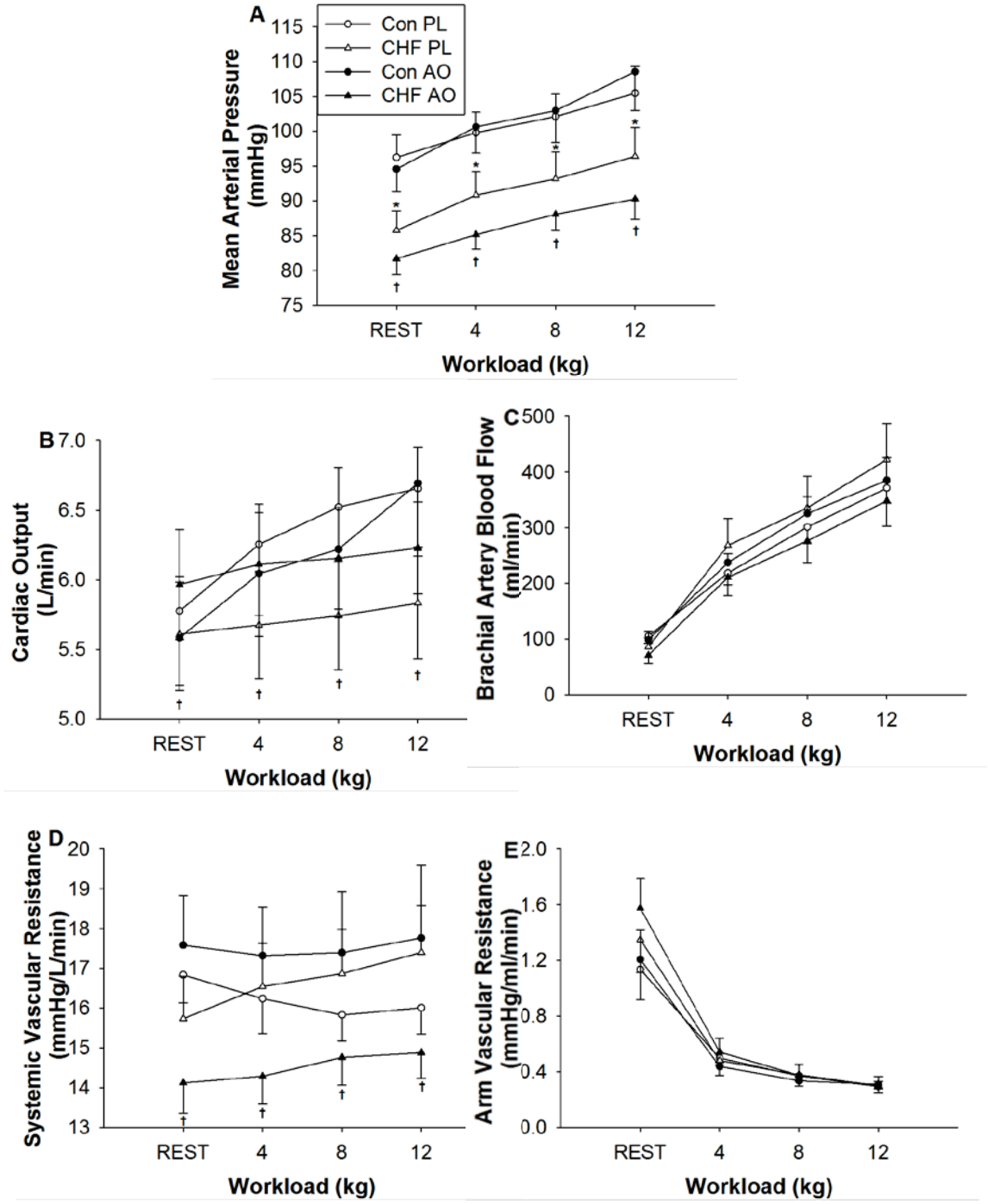

Figure 10. Central and peripheral responses to handgrip exercise in heart failure (CHF, triangle) and control subjects (Con, circle) with placebo (PL, open symbols) and the antioxidant cocktail (AOC, closed symbols). Values are mean ± SE for (A) mean arterial pressure (MAP), (B) cardiac output (CO), (C) brachial artery blood flow, (D) systemic vascular resistance, and (E) arm vascular resistance. (*) Significant difference between the controls and CHF; (†) Significant difference between PL and AOC.

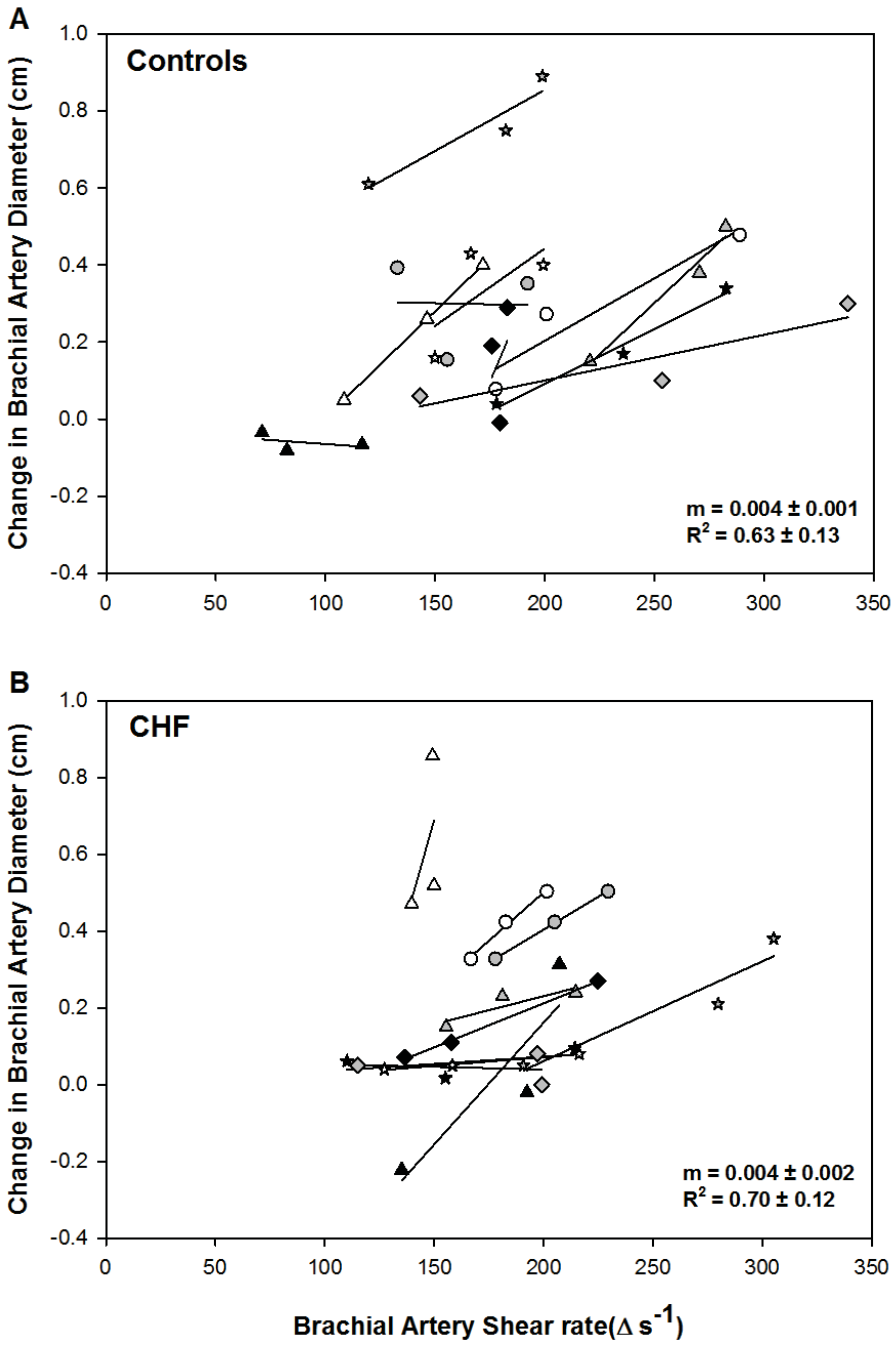

Figure 11. Individual regression plots illustrating the relationship between changes in brachial artery shear rate and the change in brachial artery diameter during 3 stages of handgrip exercise in controls (A) and CHF patients (B). There was no significant difference between the average slopes (m) of the two groups. The average strength of the relationship between shear rate and brachial artery diameter in the controls and CHF patients is represented by $R^2 \pm$ SE.

CHAPTER 5

CONCLUSION

Vascular function and blood flow regulation are important to understand and monitor as they relate to the prevalence of cardiovascular disease (4) and are even more germane in patients with CHF and following HTx. Additionally, in light of the increasing interest in isolating the physiological and mechanical contributors to the blood flow response, especially during exercise (exercise hyperemia) (10), the abnormal hemodynamic responses and exercise intolerance common in CHF and HTx patients may help to further explain the mechanisms responsible for vessel vasodilation and exercise hyperemia in both health and disease (1, 5). In CHF patients, elevated free radicals have been linked to impaired endothelial-dependent vasodilation (7, 8) and exaggerated sympathetic nerve activity particularly with exercise (6, 9). In HTx recipients, the development of high levels of oxidative stress has also been associated with endothelial dysfunction (2) and the development of cardiac transplant-associated arteriosclerosis (3). This, coupled with the highly invasive nature of heart transplantation and the effects of previous cardiac illness, lends itself to the idea that antioxidant therapy may decrease circulating levels of free radicals and may actually improve vascular function and blood flow regulation in HTx recipients. Accordingly, vascular function, blood flow regulation, and exercise-induced hyperemia were examined in healthy controls, CHF patients, and HTx recipients with varied time since surgery with and without oral antioxidant supplementation.

In the first study, we aimed to determine the central and peripheral contributions to movement-induced hyperemia in response to passive movement by comparing humans with a denervated heart (HTx) to intact controls. With this approach, we observed a four-fold reduction in the transient increase in femoral blood volume entering the leg in

response to passive limb movement in the HTx recipients compared to controls. This attenuated hyperemic response to movement in the HTx recipients was not likely due to diminished peripheral vascular function, as measured by brachial artery FMD and RH, implicating a differing central hemodynamic response as the source of disparity in leg blood flow. These findings highlight the key role of the reflex increases in HR and the associated rise in CO response as an important mechanism which contributes to movement-induced hyperemia in humans.

The second study investigated the changes in vascular function and the role of oxidative stress from health to the development of CHF, HTx, and beyond. Utilizing FMD to assess endothelium-dependent vascular function across the continuum, we documented reduced vasodilatory capacity in CHF patients, which was improved or normalized in early HTx recipients, and then an eventual decline in vascular function in the HTx recipients that were the furthest time from transplantation (> 14 yrs post-HTx) to a level that was comparable to, if not worse than, before transplantation (CHF). Interestingly, unlike the other patient groups, the acute ingestion of the AOC was able to significantly increase FMD by 55% in these > 14 yrs post-HTx recipients suggesting that free radicals, and the associated decrease in NO bioavailability, are largely responsible for their endothelial dysfunction. Somewhat surprisingly, RH, an index of microvascular function, was not different across the groups and there was no effect of the AOC, highlighting the differing physiology/pathophysiology assessed by FMD and RH. Also of significant importance to the interpretation of these data is the fact that these observations across time (e.g., time post-HTx) were not confounded by aging as the controls and all patient groups were of similar age. These findings not only highlight the

transient nature of vascular function over the course of CHF development and following HTx but also reveals the significant deterioration in endothelium-dependent vasodilation in HTx recipients who are one to two decades beyond surgery. This ultimate decline, as with the controls, appears to be a consequence of a free radically-mediated reduction in NO bioavailability.

The third study sought to better characterize the role of free radicals in regulating central and peripheral hemodynamics at rest and during exercise in patients with CHF using an oral AOC and dynamic handgrip exercise. Oxidative stress was confirmed to be significantly elevated in the CHF patients, but increased activity of the endogenous antioxidant SOD suggested some degree of compensation. Although the ingestion of the AOC further increased antioxidant capacity in both the patients and controls, only in the patients with CHF did the AOC result in a significant hemodynamic response both at rest and during exercise. Specifically, in terms of central hemodynamics, the AOC resulted in a significant reduction in MAP (~5%), an increase in CO (~10%), and a fall in systemic vascular resistance (~12%). The peripheral response to the AOC contrasted starkly with these changes with arm blood flow tending to fall and peripheral (arm) vascular resistance remaining essentially unchanged. Based upon the recognized link between free radicals and sympathetic nerve activity in patients with CHF, these data provide evidence that in patients with CHF systemic vascular resistance is, at least in part, free radically-mediated. However, this does not appear to be the result of limb or skeletal muscle-specific changes in peripheral resistance. This finding has the potential to guide future investigations targeting exercise intolerance and disease progression in this population.

Chronic heart failure is one of the most common and costly illnesses as it is a manifestation of almost every form of cardiac disease. It is of utmost importance that we understand and investigate the physiological ramifications of CHF as well as HTx as this invasive surgery is one of the only treatments for the most severe stages of the disease. In summary this research has provided significant insight into the cardiovascular consequences of CHF and HTx in terms of vascular function and hemodynamic regulation. Combined with the potential role of oxidative stress, the conclusions gleaned from these studies have broad applications for better understanding human physiology with and without cardiovascular disease as well as important therapeutic implications for improving the quality of life of these CHF patients and HTx recipients.

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